The role of NK_1 and NK_2 neurokinin receptors in the acute and sustained nociceptive activation of dorsal horn neurons.

by

Fiona E Munro

Thesis presented for the degree of Doctor of Philosophy

Department of Preclinical Veterinary Sciences,
Faculty of Veterinary Medicine,
University of Edinburgh.
1995.

DECLARATION

I hereby declare that the composition of this Thesis and the work presented in it are entirely my own with the exception of the electrophysiological studies, which were carried out in collaboration with my supervisor, Dr S. Fleetwood-Walker. Some of the material discussed in this Thesis has been presented at meetings and/or published, reprints of which are included in an appendix.

Fiona E. Munro

ACKNOWLEDGEMENTS

I gratefully acknowledge the financial support of The Medical Research Council and Glaxo Group Research for this study. There are many people who have helped me enormously during my studentship. First and foremost I would like to extend my warmest thanks to Dr Sue Fleetwood-Walker for her constant encouragement and enthusiasm over the past three years and for being such an excellent supervisor. I would also like to say a huge thankyou to Rachel Parker, Marie Young, Tracey Dickenson, Paul Heppenstall and Benji for making 'lab 97' such a fun place to work, for their friendship and support and for helping to regularly distract me from my studies.

I am extremely grateful to Dr Rory Mitchell for his expert advice and guidance with my biochemical studies and for help in the selection of appropriate compounds for my project. I would also like to have the opportunity to thank all the members of Rory's team for letting me loose in the lab once a week; special thanks to Pauline Sim and Melanie Johnson for all their invaluable help.

My sincere thanks go to Dr Phil Birch at Glaxo Group Research, Ware, for his continual support and encouragement; I would also like to thank the 'Pharmacology 1' team at Glaxo for making my time there so enjoyable, I am especially grateful to Jo Bowers, Alan Wheeldon and Sue Harrison for their excellent guidance and never ending readiness to help.

I would like to extend my appreciation to Bobbie Rosie from the MRC Brain Metabolism Unit for her expert advice in the technique of *in situ* hybridisation histochemistry and to Dr Vince Molony for his support and continued interest in my studies. There are a number of people from whom I have received excellent technical assistance:- I would especially like to thank Colin Warwick (and Rowan) in the Photography and Graphics department for his skill, patience and good humour; Gordon Goodall, Judith Watson and Kate Rennie for all their expert help, fun and good music in Histology. Likewise, special thanks to Ian Stuart for his patience and help with the computer software. I also greatly appreciate the valued technical help of Anne Stirling-Whyte, Suzanne Sellar and Elaine Chalmers in the secretarial department; Terry Dyter and Andy MaCrae in the electronics workshop; Jimmy Brown and Graham Moodie in the mechanical workshop; Maggie Matheson and Callum Paterson from the Wellcome Animal Research Unit as well as Lucy Mitchell and Maxine Palagi for help in the laboratory.

I would like to specifically thank all my friends and colleagues in the Department of Preclinical Vet Sciences for being so helpful and friendly and making my time there so enjoyable. Finally, I should like to thank my parents for their moral support and Gregor for encouragement, understanding and constant support over the years.

CONTENTS

DECLARATION **2**
ACKNOWLEDGEMENTS **3**
ABSTRACT **9**
ABBREVIATIONS **11**

CHAPTER 1: General Introduction

1.1. Introduction **14**
1.2. Cutaneous Sensory Afferents and Receptors **15**
1.2.1. Cutaneous Sensory Afferents 15
1.2.2. Cutaneous Receptors 15
1.3. The Dorsal Horn of the Spinal Cord **21**
1.3.1. Laminar Organisation 21
1.3.2. Cutaneous Primary Afferent Input into the Dorsal Horn 24
1.3.3. Classification of Dorsal Horn Neurons 26
1.4. Ascending Somatosensory Projection Tracts **28**
1.4.1. The Spinothalamic Tract 28
1.4.2. The Spinoreticular Tract 30
1.4.3. The Spinocervical Tract 30
1.4.4. Postsynaptic Dorsal Columns 31
1.4.5. The Spinomesencephalic Tract 32
1.5. Tachykinins **32**
1.5.1. Synthesis of Tachykinin Peptides 32
1.5.2. Distribution of Tachykinins in the Spinal Cord and Dorsal Roots 37
1.6. Tachykinin Receptors **43**
1.6.1. Distribution of Tachykinin Receptors in the Spinal Cord 43
1.7. The Involvement of Tachykinins in Acute and Sustained Nociception **48**
1.7.1. Acute Nociceptive States and the Possible Involvement of Tachykinins in Their Production 48
1.7.2. Sustained Nociceptive States and the Possible Involvement of Tachykinins in Their Production 57
1.8. Biochemical Changes in Spinal Cord During Sustained Nociceptive Input **64**
1.9. Molecular Mechanisms of Neuronal Plasticity Induced by Synaptic Input **67**
1.9.1. Noxious Stimulus-Induced Expression of c-fos 67
1.9.2. Consequences of c-fos Expression 70
1.10. Aims of Current Work 74

CHAPTER 2 : Electrophysiological Examination of Sensory Responses in Laminae III-V Rat Dorsal Horn Neurons

2.1 Aims **75**
2.2 Materials **75**
2.3 Methods **76**
2.3.1 Animals 76
2.3.2 Surgery 76
2.3.3 Recording and Ionophoresis 81
2.3.4 Histological Determination of Laminar Location 82
2.3.5 Quantification of Sensory Responses 82
2.3.6 Analysis 85
2.4 Results **88**
2.4.1 Characteristics and Receptive Field Properties of Neurons 88
2.4.2 Current and Vehicle Controls 91
2.4.3 Control Responses to Sensory Stimuli 91
2.4.4 Effects of Ionophoretically Applied Neurokinin Antagonists 91
2.4.5 Effects of Ionophoretically Applied Neurokinin Agonists 104
2.4.6 Co-Ionophoresis of NK_1 Agonist and Antagonist 109
2.4.7 Co-Ionophoresis of NK_2 Agonist and Antagonist 109
2.5 Discussion **110**

CHAPTER 3: The Effects of Neurokinin Antagonists on Mustard Oil-Evoked Activation of Dorsal Horn Neurons

3.1 Aims **116**
3.2 Materials **116**
3.3 Methods **117**
3.3.1 General Methods 117
3.3.2 Application of Mustard Oil and Quantification of Drug Responses 117
3.3.3 Analysis 118
3.4 Results **118**
3.4.1 Characteristics and Receptive Field Properties of Neurons 118
3.4.2 Control Responses 119
3.4.3 Cutaneous Application of Mustard Oil 119
3.4.4 Mustard Oil-Induced Peripheral Effects 120
3.4.5 Effect of NK_2 Antagonists on Mustard Oil-Induced Activity 120
3.4.6 Effect of NK_1 Antagonists on Mustard Oil-Induced Activity 132
3.5. Discussion **135**

CHAPTER 4: Electrophysiological Examination of Evoked Sensory Responses in Laminae III-V Dorsal Horn Neurons Before and After Application of Mustard Oil.

4.1 Aims **140**
4.2 Materials **140**
4.3 Methods **141**
4.3.1 General Methods 141
4.3.2 Quantification of Sensory Responses Before and After Application of Mustard Oil 141
4.3.3 Analysis 142
4.4 Results **143**
4.4.1 Characteristics and Receptive Field Properties of Neurons 143
4.4.2 Control Responses to Sensory Stimuli 144
4.4.3 Effects of Ionophoretically Applied Neurokinin Antagonists on Sensory Responses Before and After Application of Mustard Oil 144
4.5 Discussion **159**

CHAPTER 5 : The Role of Protein Kinase C (PKC) in Sustained Nociceptive Responses of Rat Dorsal Horn Neurons.

5.1 Aims **166**
5.2 Materials **166**
5.3 Methods - Electrophysiology **167**
5.3.1 General Methods 167
5.4 Methods - [^{3}H]phorbol 12,13-dibutyrate Binding Studies **167**
5.4.1 Animals 167
5.4.2 Drugs 167
5.4.3 Experimental Protocol 167
5.4.4 Analysis 171
5.5 Results - Electrophysiology **172**
5.5.1 Characteristics and Receptive Field Properties of Neurons Tested 172
5.5.2 Current and Vehicle Controls 173
5.5.3 Effect of Cutaneous Application of Mustard Oil 173
5.5.4 Effect of Ionophoretically Applied PKC Inhibitors 174
5.6 Results - PDBu Binding Studies **177**
5.6.1 The Effect of Mustard Oil on PDBu Binding in Rat Spinal Cord 177
5.6.2 The Effect of SR 48968 on Mustard Oil-Induced PKC Translocation in Rat Spinal Cord 177
5.7 Discussion **182**

CHAPTER 6 : Mustard Oil-Induced c-fos mRNA Expression in Rat Spinal Cord

6.1 Aims **188**
6.2 Materials **188**
6.3 Methods **189**
6.3.1 Animals 189
6.3.2 Tissue Preparation 189
6.3.3 Subbing Slides 193
6.3.4 Cutting Sections 193
6.3.5 Probe Labelling 196
6.3.6 Hybridisation 199
6.3.7 Apposing Slides 200
6.3.8 Developing and Staining Slides 200
6.3.9 Analysis 200
6.4 Results **202**
6.4.1 Detection of c-fos mRNA with ISHH; Confirmation of Technique 202
6.4.2 Control Studies 202
6.4.3 The Effect of Mustard Oil on c-fos Expression 209
6.4.4 The Effect of RP 67580 on the Mustard Oil-Facilitated c-fos mRNA Expression 213
6.4.5 The Effect of SR 48968 on the Mustard Oil-Facilitated c-fos mRNA Expression 217
6.5 Discussion **220**

CHAPTER 7 : Behavioural Experiments

7.1 Aims **228**
7.2 Materials **228**
7.3 Methods **228**
7.3.1 Animals 228
7.3.2 Drug Administration 228
7.3.3 Nociceptive Testing in Non-Carrageenan Treated Animals 229
7.3.4 Nociceptive Testing in Carrageenan Treated Animals 235
7.3.5 Analysis 238
7.4. Results 241
7.5.1 Control Paw-flick and Tail-flick Responses 241
7.5.2 Nociceptive Testing in Non-Carrageenan Treated Animals 241
7.5.3 Nociceptive Testing in Carrageenan Treated Animals 251
7.6 Discussion 255

CHAPTER 8 : Conclusions 263

BIBLIOGRAPHY 268

APPENDIX : Publications Arising From Research 314

ABSTRACT

The tachykinins substance P (SP) and neurokinin A (NKA) can be released from fine somatosensory afferents into the spinal cord by noxious cutaneous stimuli. This study assessed the role of their respective NK_1 and NK_2 receptors and some putative intracellular mediators in both acute and sustained nociceptive inputs to dorsal horn neurons:

(a) In anaesthetised rats, extracellular recordings were made from laminae III-V multireceptive neurons. The ionophoretic administration of NK_1 antagonists L-668,169, GR 82334 and [D-Pro^4,D-Trp^{7,9,10}Phe^{11}]substance P-(4-11) failed to influence neuronal responses to noxious pinch or heat, but often enhanced responses to innocuous brush, whilst the NK_2 antagonist L-659,874 inhibited responses to noxious heat, but not pinch or brush. Selective NK_1 and NK_2 receptor agonists, [N-acetyl-Arg^6,Sar^9,Met(O$_2$)^{11}]SP$_{6-11}$ and GR 64349 respectively, both excited dorsal horn neurons. The contribution of NK_1 and NK_2 receptors to sustained neuronal activity induced by peripheral application of the C-fibre selective algogen mustard oil was then investigated. The mustard oil-induced activity was inhibited by the selective NK_2 receptor antagonist L-659,874 but not by the selective NK_1 antagonists L-668,169 or GR 82334. The role of NK_1 and NK_2 receptors was further examined in the central neuronal sensitisation of both innocuous and noxious inputs, induced by mustard oil. Prior to mustard oil application, the NK_1 antagonists RP 67580 and GR 82334 selectively enhanced neuronal responses to innocuous brush, whilst NK_2 antagonists SR 48968 and L-659,874 selectively inhibited responses to noxious heat, however, following repeated mustard oil application, the facilitated neuronal responses to brush and heat were blocked by both selective NK_1 and NK_2 antagonists.

(b) Evidence for a role of protein kinase C (PKC) in mediating sustained nociceptive responses of rat dorsal horn neurons was provided by the blockade of mustard oil-, but not brush-evoked neuronal activation by the PKC inhibitors GF 109203X and chelerythrine and by SR 48968-sensitive subcellular translocation of [^{3}H]phorbol 12,13-dibutyrate binding sites ipsilateral to mustard oil stimulation.

(c) *In Situ* hybridisation histochemistry (ISHH) demonstrated that expression of c-fos mRNA, induced in the superficial dorsal horn by peripheral application of mustard oil was inhibited by systemic administration of both RP 67580 and SR 48968.

(d) The effects of intrathecally-applied NK_1 and NK_2 antagonists were assessed on thermally-evoked tail-flick and paw-flick behavioural responses. GR 82334 and L-659,874 had no effect alone, but in combination inhibited paw-flick. After inflammation induced by intraplantar injection of carrageenan, each was effective individually.

These results provide evidence that spinal NK_2 receptors are involved in mediating both acute and sustained nociceptive transmission, probably acting through phosphoinositide hydrolysis and stimulation of protein kinase C (PKC). However, evidence for NK_1 receptor involvement was only obtained in sustained or inflammatory models of nociceptive transmission in the spinal dorsal horn.

ABBREVIATIONS

AA	arachidonic acid
BB	bombesin
BSA	bovine serum albumin
CC	column of Clarke
CCI	chronic constriction injury
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CP-96,345	[(2S,3S)-cis-2-(diphenylmethyl)-N-[2-methoxyphenol)-methyl]-1-azabicyclo[2.2.2]octan-3-amine]
DAG	diacylglycerol
DEP	diethylpyrocarbonate
dH_2O	distilled water
DLH	D,L-homocysteic acid
DNA	deoxyribonucleic acid
DPFL	dorsal paw-flick latency
drg	dorsal root ganglion
EAAs	excitatory amino acids
ENK	enkephalin
fig	figure
GAL	galanin
GF109203X	2-[1-(3-dimethylaminopropyl)-indol-3yl]-3-(indol-3-yl)maleimide
Glu	glutamate
GPFL	glabrous paw-flick latency
GR 64349	[Lys^3,Gly^8-R-γ-lactam-Leu^9]neurokinin A-(3-10)
GR 82334	[D-Pro^9[spiro-γ-lactam]-Leu^{10},Trp^{11}]physalaemin-(l-11)
HCl	hydrochloric acid
[^{3}H]PDBu	[20-^{3}H(N)]-phorbol,12,13-dibutyrate
HRP	horseradish peroxidase
HT	high threshold
5-HT	5-hydroxytryptamine

Hz	hertz
[^{125}I]-BH-NKA	^{125}I-Bolton-Hunter neurokinin A
[^{125}I]-BH-SP	^{125}I-Bolton-Hunter substance P
IL	intermedio-lateral nucleus
IM	intermedio-medial nucleus
IR	infrared
i.p.	intraperitoneal
i.t.	intrathecal
i.v.	intravenous
IP_3	inositol (1,4,5 P_3) triphosphate
ISHH	*in situ* hybridisation histochemistry
L-659,874	acetyl-Leu,Met,Gln,Trp,Phe-Gly-NH_2
L-659,877	cyclo(Gln,Trp,Phe,Gly,Leu,Met)
L-668,169	cyclo(Gln,D-Trp,Me-Phe,(R)Gly[ANC-2]Leu,Met)$_2$
LISS	Lissauer's tract
LM	lateromedial nucleus
LSN	lateral spinal nucleus
LT	low threshold
LTP	long term potentiation
MEN 10207	[Tyr^5,D-Trp^{6,8,9},Arg^{10}]neurokinin A-(4-10)
MEN 10376	[Tyr^5,D-Trp^{6,8,9},Lys^{10}]neurokinin A-(4-10)
mg; µg	milligram; microgram
min(s)	minute(s)
ml; µl	millilitre; microlitre
mm; µm	millimetre; micrometre
mRNA	messenger ribonucleic acid
nA	nanoamp
NaCl	sodium chloride
NKA	neurokinin A
NKB	neurokinin B
NMDA	N-methyl-D-aspartate
nmol	nanomole
NPK	neuropeptide K
NPγ	neuropeptide γ
NS	nociceptive specific
$P<0.05$	probability less than 5%
PAG	periaqueductal grey

PBS	phosphate buffered saline solution
PI	phosphoionositide
PKC	protein kinase C
PLC	phospholipase C
PPT	preprotachykinin
PSB	pontamine sky blue
PSDC	post-synaptic dorsal column
R396	Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2
RNA	ribonucleic acid
RP 67580	2-[1-imino-2-(2-methoxyphenyl)ethyl]-7,7diphenyl-4 perhydroisoindolone (3aR,7aR)
RP 68651	2-[1-imino-2-(2-methosyphenyl)ethyl]-7,7diphenyl-4 perhydroisoindolone (3aS,7aS)
R/s	response per second
Sar-9	[N-acetyl-Arg^6,Sar^9,$Met(O_2)$]SP_{6-11}
SCT	spinocervical tract
sec.	second
s.e.m.	standard error of mean
SG	substantia gelatinosa
SMT	spinomesencephalic tract
SP	substance P
SPOMe	[$Met\text{-}OMe^{11}$]SP
SR 48968	(S)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl] benzamide
SR 48965	(R)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl] benzamide
SRT	spinoreticular tract
SS	somatostatin
SSC	standard sodium citrate
STT	spinothalamic tract
TFL	tail-flick latency
UV	ultra violet
vol.	volume
VRP	ventral root potential
WDR	wide dynamic range
WGA	wheatgerm agglutinin
wt.	weight

CHAPTER 1:
General Introduction

1.1 INTRODUCTION

In the early 19th Century, two opposing theories were proposed to explain how the nervous system differentiates between the various forms of sensory experience, these were the 'Intensity Theory' and the 'Specificity Theory':-

Goldscheider (1884) formulated the Intensity Theory which maintained that pain has no separate receptors, but is produced in the nervous system as a result of summation of impulses excited by the application of noxious pressure or temperature to the skin. Thus weak stimulation would excite the cutaneous sensory nerve endings to a low level, regarded as normal, but as stimulation increased it would evoke pain.

This view was challenged by a strong body of opinion in favour of the 'Specificity Theory'. Charles Bell was the first to suggest that there was 'specificity' in the cutaneous nervous system (see Sinclair, 1967). However, this theory is generally attributed to Muller derived from his law of 'specific sensory energies'. Muller proposed that activity in a particular nerve would give rise to a specific sensation regardless of the sensory stimulus used (Muller, 1842). Evidence supporting this theory came from Blix (1884) who discovered that in normal skin, stimulation of tiny 'spots' would react preferentially to touch, cold, warm and pain. In 1895, Von Frey suggested that under the skin of each of these sensory spots there lay one or more sense organs specialised to respond to a particular type of stimulus. Von Frey, supporting the specificity theory proposed that hair follicle endings in hairy skin and Meissner's corpuscles in glabrous skin would respond to touch, Krause's end bulbs to cold, Ruffini endings to warmth and free endings to pain. However, it has since been discovered that Ruffini endings are identified with slowly adapting mechanoreceptors (Chambers *et al*, 1972); Krause's end bulbs are a variety of rapidly adapting mechanoreceptors (Iggo and Ogawa, 1977) and thermal sensitivity depends on free nerve endings (Andres and During, 1973).

In 1965, Melzack and Wall proposed the 'Gate Control Theory' for nociceptive transmission within the spinal cord, suggesting that the spinal cord substantia gelatinosa (SG) cells act as a gating mechanism between primary afferents and higher brain areas. They proposed that the SG cells modulate

afferent transmission before it affects the central transmission (T) cells which in turn relay sensory information to higher levels of the brain. Activity in large and small afferent fibres results in negative and positive feedback respectively onto the substantia gelatinosa cells. Melzack and Wall stated that for pain to occur, there would have to be (a) ongoing activity in small myelinated and unmyelinated fibres, (b) stimulus-evoked activity and (c) a relative balance of activity in large and small afferent fibres. The tonic activity in small myelinated and unmyelinted afferent fibres would keep the gate partly open, whereas if large myelinated fibres were activated the gate would close, limiting the discharge of T cells.

There have been many criticisms of the Gate Control Theory (Nathan, 1976; Cervero and Iggo, 1980) and although aspects of its original configuration may not now be tenable, the idea of the substantia gelatinosa acting as a centre of integration and regulation remains a useful hypothesis.

1.2 CUTANEOUS SENSORY AFFERENTS AND RECEPTORS

1.2.1 Cutaneous Sensory Afferents

In cutaneous nerves the Aαβ class comprises the largest group of fibres with the fastest conduction velocities (30-100 m/s) and innervate corpuscular endings or hair follicle receptors. The small myelinated fibres belong to the Aδ or class III group which supply hair follicle receptors and mechanical nociceptors. They conduct at 4-30 m/s, whereas the unmyelinated fibres (C-fibres) have the smallest diameters which conduct at less than 2.5 m/s, innervating mechanical, thermal and polymodal nociceptors as well as sensitive mechanoreceptors (hair), warm receptors and are also present in sympathetic efferents. (Zimmerman, 1968; Iggo, 1974). Most of the information about the nature and properties of nociceptive afferent units has come from recordings of the activity of single afferent fibres. It was Adrian (1931) and Zotterman (1939) who first demonstrated that noxious stimulation evoked activity in unmyelinated C-fibres and in slowly conducting myelinated Aδ fibres. Torebjork and Hallin (1973, 1974) have confirmed these findings in conscious human studies, where they demonstrated that the subject only felt pain when the cutaneous nerve was stimulated at C-fibre but not at A-fibre intensity

1.2.2 Cutaneous Receptors

The terminal portions of the primary afferent neuron lying in the peripheral tissue (skin and the adjacent subcutaneous connective tissue) constitute

the receptive or dendritic part of the cell (Bodian, 1962), which receive the stimulus and translate it into nerve impulses which propagate along the afferent fibre. As previously mentioned, on account of the methods developed by Adrian and Zotterman in 1926, there has been rapid advancement in our understanding of the nature and properties of individual afferent fibres. This method and others have enabled a detailed study of cutaneous receptors which has generated three sub-classes, mechanoreceptors, thermoreceptors and nociceptors. (Iggo, 1966; Burgess and Perl, 1973; Hensel, 1973). See Figure 1.1 for a schematic representation of the neuronal organisation of and afferent input of the superficial dorsal horn.

(a) Mechanoreceptors

Cutaneous mechanoreceptors are most readily activated by mechanical changes to the skin, they can also be affected by thermal changes (Iggo, 1966) but this is thought to be insignificant physiologically (Duclaux and Kenshalo, 1972). Burgess and Perl (1973) have classified 3 classes of cutaneous mechanoreceptors on their ability to detect position, velocity or transients of stimulus.

Receptors Detecting Cutaneous Displacement and Velocity. There are two sub-classes of receptors detecting cutaneous displacement and velocity which are (i) slowly and (ii) rapidly adapting cutaneous mechanoreceptors (Iggo, 1966; Brown and Iggo, 1967).

Receptors Detecting Velocity. There are at least 5 kinds of cutaneous sensory receptors that detect velocity. Four are found in hairy skin (G_2 hair follicle receptors, D hair follicle receptors, field receptors and C mechanoreceptors) and one in glabrous skin (Meissner's corpuscle), (Brown and Iggo, 1967; Burgess and Perl, 1973).

Receptors Detecting Transient Movement. There are two types of cutaneous receptors that are designed to detect transients : G_1 Hair Follicle Receptors and Pacinian Corpuscles, both of which activate $A\alpha\beta$ primary afferent fibres (Burgess *et al*, 1968).

(b) Thermoreceptors

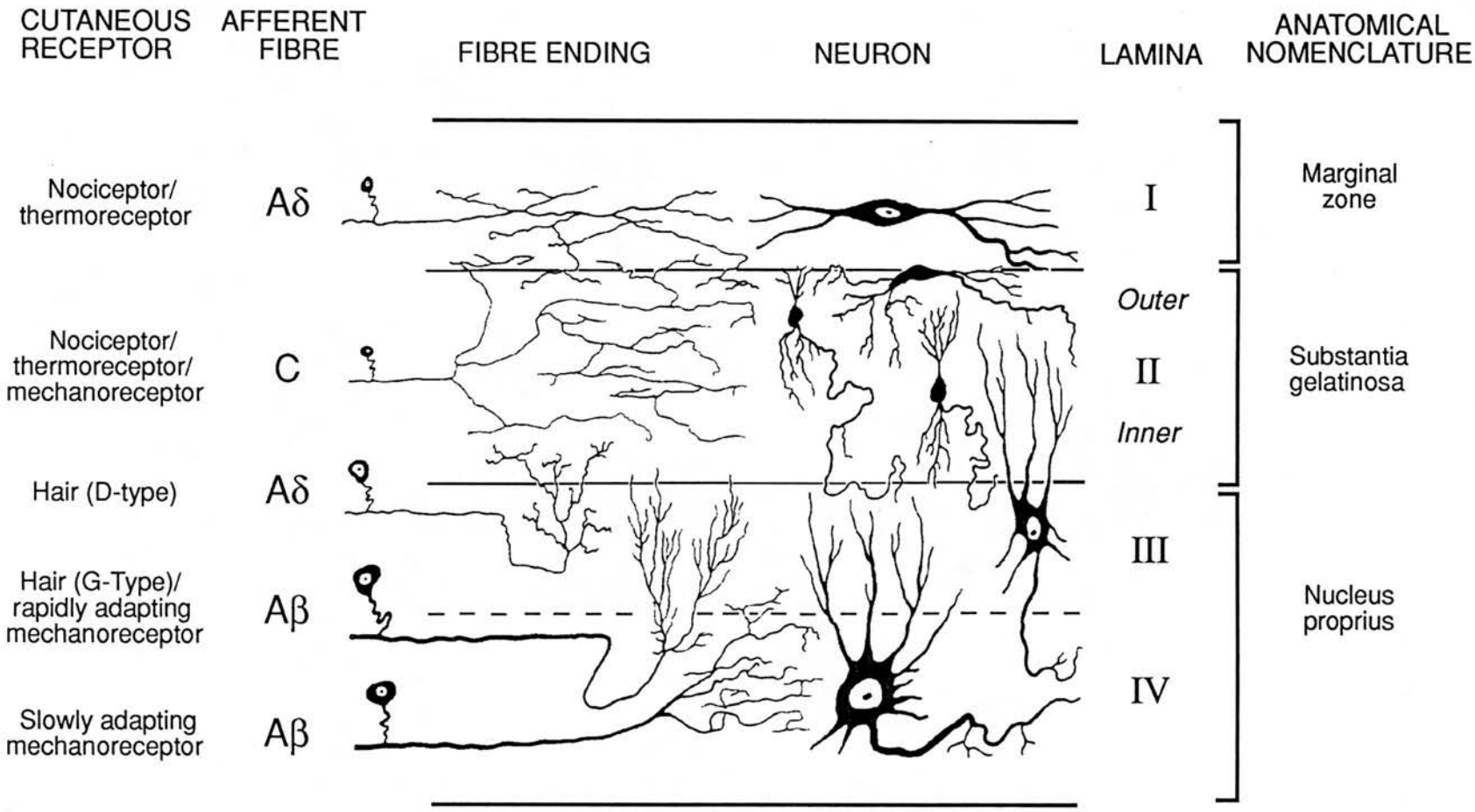
Hensel (1973) described the neurophysiological properties of cutaneous thermoreceptors as having (a) a static discharge at constant temperatures, (b) a dynamic response to temperature changes and (c) no excitatory response to mechanical stimuli within reasonable intensity. There are 2 kinds of cutaneous

Figure 1.1

Schematic diagram of the neuronal organisation of, and afferent input to the superficial dorsal horn.

The diagram represents an imaginary transverse section of the dorsal horn and illustrates the afferent fibre endings and neuronal elements present in the first four laminae of the dorsal horn. To the left of the diagram the types of afferent fibre and relevant receptor groups associated to them are listed. Fibre endings in the dorsal horn are schematised diagrams taken from published morphological studies. Neurons in the diagram represent standard types of neuron in the superficial dorsal horn. The following types have been illustrated: *(from top to bottom)* a marginal cell, an SG limiting cell, two SG central cells and two neurons of the nucleus proprius, the most superficial of which has dendrites penetrating lamina II. Indicated at the right of the diagram are the laminar division of the superficial dorsal horn and corresponding nomenclature.

(Taken from Cevero and Iggo, 1980)


CUTANEOUS RECEPTOR
AFFERENT FIBRE
FIBRE ENDING
NEURON
LAMINA
ANATOMICAL NOMENCLATURE
Nociceptor/ thermoreceptor
Aδ
Nociceptor/ thermoreceptor/ mechanoreceptor
C
Hair (D-type)
Aδ
Hair (G-Type)/ rapidly adapting mechanoreceptor
Aβ
Slowly adapting mechanoreceptor
Aβ
I
Outer
II
Inner
III
IV
Marginal zone
Substantia gelatinosa
Nucleus proprius

receptors which signal innocuous changes in skin temperature, these are warm and cold receptors.

Cold Receptors may be either myelinated (Hensel and Zotterman, 1951; Iggo, 1969) or unmyelinated (Iggo, 1959; Douglas *et al*, 1960) and are found in both hairy and glabrous skin (Iggo, 1969).

Warm Receptors are generally thought to be unmyelinated (Iggo, 1959; Hensel *et al*, 1960). However, Hensel (1966) has reported studies demonstrating myelinated warm fibres. Warm receptors are active at normal body temperature, their discharge slows with cooling of the skin and increases with heating. However, they are silenced by noxious levels of radiant heat (LaMotte and Campbell, 1978).

(c) Nociceptors

Nociceptors were first described by Sherrington (1900) as sensory endings that respond to stimuli that threaten or actually damage tissue. Since then, sub-populations of primary afferent fibres that respond to excessive mechanical or thermal stimuli have been described in animals (Burgess and Perl, 1967; Bessou and Perl, 1969) and in man (Van Hees and Gybels, 1972). There are two main groups of cutaneous nociceptors, the Aδ mechanical nociceptor and the C-polymodal nociceptor (see Figure 1.1 for a schematic diagram).

Aδ Mechanoreceptors are nociceptors associated with Aδ fibres which are only excited by noxious mechanical stimulation (Burgess and Perl, 1967; Burgess *et al*, 1968; Georgopoulis, 1976). They are referred to as high threshold mechanoreceptors although thresholds do vary. They have reasonably large receptive fields (1-2 cm diameter) comprising of several sensitive zones separated by areas where mechanical stimulation is without effect. They are not activated by thermal or chemical stimuli (Burgess and Perl, 1967).

Certain Aδ nociceptors can be sensitised by repeated applications of noxious heat to their receptive fields (Fitzgerald and Lynn, 1977; Lynn and Carpenter, 1982; Treede *et al,* 1992). However, many Aδ units will fatigue after a repetitive application of the stimulus and if this is intense enough, cessation of firing can occur (Fitzgerald and Lynn, 1977). Although rare, specific mechanoreceptors associated with unmyelinated fibres have been described (Georgopoulis, 1976).

C-Polymodal Nociceptors. Bessou and Perl (1969) originally described polymodal nociceptors by their responsiveness to intense thermal and mechanical stimuli as well as to irritant chemicals. In the absence of stimulation, these

receptors do not produce any spontaneous activity, but they respond well to noxious mechanical, thermal and chemical stimuli (Bessou and Perl, 1969). Their receptive field area is generally small e.g. one digit of the ipsilateral paw, although a degree of overlap exists between the receptive fields of several afferent fibres (Torebjork, 1974). The most effective mechanical stimuli are those of greatest noxious intensity applied by pointed objects and effective thermal stimuli are noxious heat (greater than 45°C) (Bessou and Perl, 1969).

The nociceptors will increase their discharge with intensity of stimulation, after a large initial discharge, the response adapts and settles to a lesser level which can continue after cessation of the stimulus (Adrian and Zotterman, 1926; Torebjork and Hallin, 1974; Croze *et al,* 1976). Another characteristic of C polymodal nociceptors consists of the appearance of 'fatigue' following the repetition of a noxious stimulus to the same point on the receptive field (Bessou and Perl, 1969; Van-Hees and Gybels, 1972; Torebjork and Hallin, 1974; Kumazawa and Perl, 1977; Lynne and Carpenter, 1982).

C polymodal nociceptors can be sensitised by a prior nociceptive thermal stimulus. Sensitisation can be manifest by several changes, a reduction in threshold, a reduced response latency to a given thermal stimulus and the appearance of spontaneous activity (Bessou and Perl, 1969; Croze *et al,* 1976; Kumazawa and Perl, 1977; Lynn and Carpenter, 1982). This sensitisation is more marked in hairy than glabrous skin (Campbell and Meyer, 1983).

Other Nociceptors have been described including:-

C Mechanical Nociceptors- C mechanical nociceptors have been described by Iggo, 1960; Bessou and Perl, 1969; Beck *et al,* 1974; Georgopoulis, 1976; Kumazawa and Perl, 1977; LaMotte and Campbell, 1978; Lynn and Carpenter, 1982). Intense mechanical stimuli can activate the cold and warm thermoreceptors although their responses are often only moderate and variable. It is possible that some of these receptors are the same as the C cold nociceptors (Bessou and Perl, 1969).

Aδ Mechanoheat Nociceptors- Aδ Mechanoheat Nociceptors respond well to both mechanical and thermal testing (Beck *et al,* 1974; Georgopoulis, 1976; Kumazawa and Perl, 1977; Treede *et al,* 1992), although the threshold for thermal testing is much higher (51-53°C) than that of C polymodal nociceptors (LaMotte *et al,* 1983). Aδ and C cold nociceptors respond both to extreme cold and to intense mechanical stimulation (Iggo, 1959; Bessou and Perl, 1969; Georgopoulis, 1976).

1.3 THE DORSAL HORN OF THE SPINAL CORD.

1.3.1 Laminar Organisation

In 1952, Rexed gave the first comprehensive and systematic account of the histology of the spinal cord grey matter. This cytoarchitectonic laminar scheme of the cat spinal cord presented by Rexed (1952,1954) has been reproduced for the rat by Molander (1984,1986) (see Figure 1.2) and concentrates on the shapes, sizes, density and distribution of the neuronal somata. It does not take into consideration the dendritic trees of neurons or axonal projections and terminations. There are 9 distinguishable cell layers (laminae) in the cord together with a region around the central canal (lamina X) described by Rexed and extend through the rostro-caudal length of the cord (see Figure 1.2). The dorsal horn consists of :-

Lamina I is the most dorsal of the layers, covering the dorsal surface of the dorsal horn it bends around its apex and tapers down the lateral side. It is a relatively thin layer of predominantly small cells, although a few larger marginal cells can be found overlying the gelatinosa with their major dendrites arranged horizontally (Szentagothai, 1964; Scheibel and Scheibel, 1968; Waldeyer, 1988). Although the smaller cells are more numerous, they have not been extensively studied.

Lamina II lies immediately ventral to lamina I, as a well-defined layer running from the medial, across and down the lateral side, and is known as the substantia gelatinosa (Rolando, 1824). The substantia gelatinosa (SG) displays closely packed small cells in the outer zone and a more loose arrangement in the inner zone (Cervero and Iggo, 1980). Rexed (1952,1954) has equated the lamina II layer with the substantia gelatinosa and it can be subdivided into laminae II_o and II_i (Gobel *et al,* 1980). Lamina II_o, the dorsal or outer part of the SG contains stalk cells whose dendrites form a cone emanating from the cell body and passing ventrally through laminae II-IV. Islet cells are located in lamina II_i (the inner or ventral part of the SG) and dendritic trees extend from these cells to cover the entire width of lamina II, (Gobel, 1978).

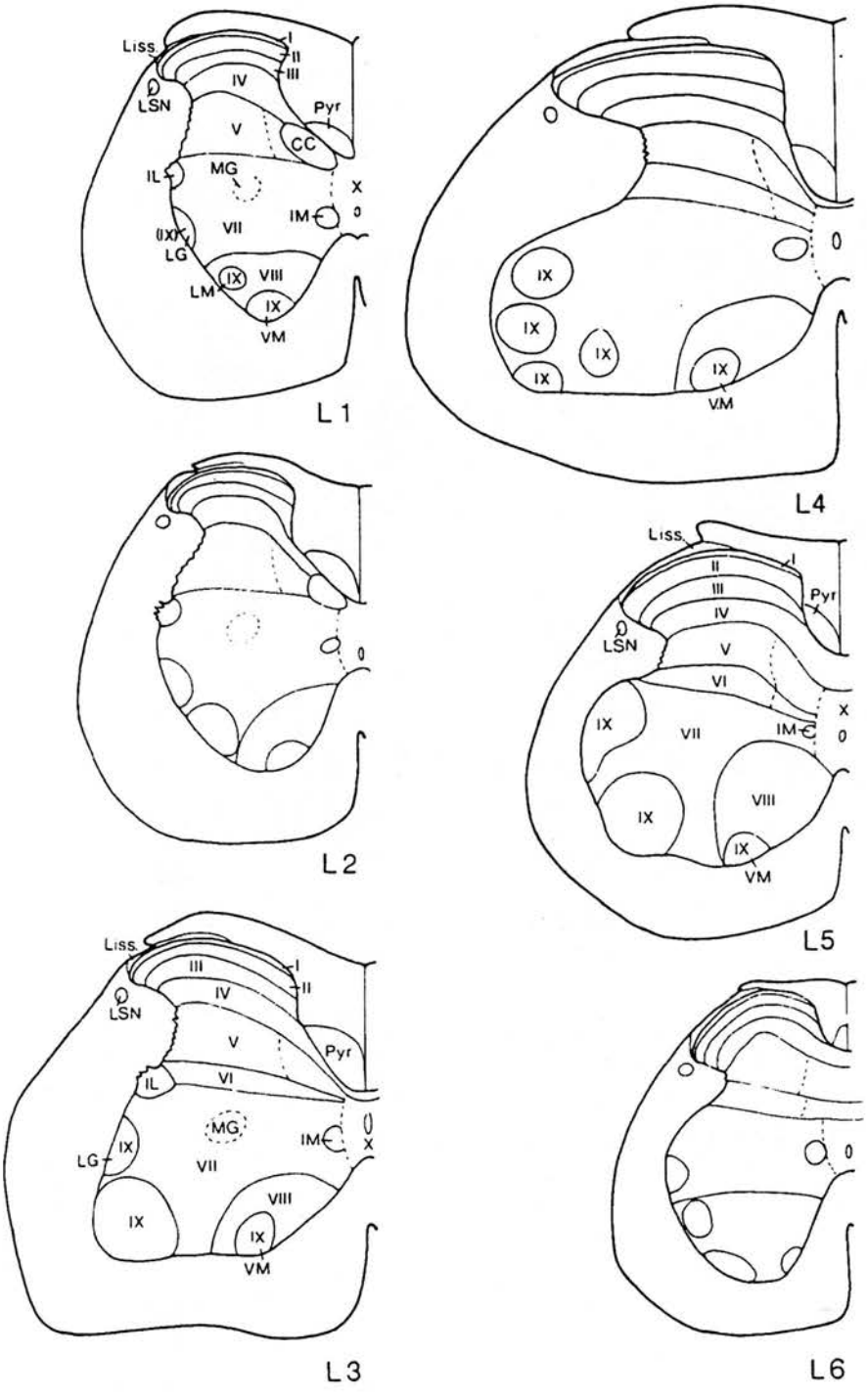
Lamina III lies ventral to lamina II and is characterised by densely aggregated cells (Molander *et al,* 1984), being slightly larger and more widely spaced than in lamina II. (Rexed, 1952), however the border between laminae II and III is indistinct with a slightly transitional character.

Figure 1.2

Schematic drawings of the cytoarchitectonic subdivisions of sements L1-L6 rat spinal cord laminae.

CC, column of Clarke; IL, intermedio-lateral nucleus; IM, intermedio-medial nucleus; LSN, lateral spinal nucleus; Liss, Lissauer's tract; LG, lateral group of large cells in the dorso-lateral part of the ventral horn; LM, latero-medial nucleus; MG, medial group of large neurons in the intermediate zone. Note that LG, LM and VM are parts of lamina IX.

(Taken from Molander *et al,* 1984)


Liss.
I
II
III
IV
LSN
Pyr
V
CC
MG
IL
X
IM
VII
(IX)
LG
IX
VIII
LM
IX
VM
L1
IX
IX
IX
IX
VM
L4
L2
Liss.
II
III
IV
Pyr
LSN
V
VI
X
IX
VII
IM
VIII
IX
IX
VM
L5
Liss.
III
I
II
IV
LSN
V
Pyr
IL
VI
MG
IM
X
LG
IX
VII
VIII
IX
IX
VM
L3
L6

Lamina IV forms the upper part of the base of the head of the dorsal horn. It is a relatively thick layer which extends across the dorsal horn with neurons of varying sizes (Molander *et al,* 1984), easily distinguished from lamina III, where the neuron size is more uniform. There are very large cells present which have dendrites which pass laterally, dorsally and medially, but with a general longitudinal orientation. Both Spinocervical Tract (SCT) and Postsynaptic Dorsal Column (PSDC) projection cells are present in laminae IV.

Lamina V extends as a thick band across the narrowest part of the neck of the dorsal horn. The presence of the lateral reticulated area with numerous myelinated fibres makes lamina V distinctive and in comparison to laminae IV, it is characterised by even larger cells (Molander *et al,* 1984) with a different shape of dendritic field.

Lamina VI exists only in the cervical and lumbosacral enlargements of the spinal cord (Rexed, 1952) and cells there were only occasionally encountered in the present study. The cells are smaller, have a more homogenous distribution than those in lamina III and are darker than those of lamina V (Molander *et al*, 1984).

1.3.2 Cutaneous Primary Afferent Input into the Dorsal Horn

The morphology of the primary afferent input into the various laminae of the dorsal horn is very important to understanding spinal sensory mechanisms. There are various techniques used for investigation, classically these have involved mainly Golgi and degeneration techniques (Fitzgerald, 1984). However more recently one of the most successful techniques is that of intra-axonal injection of the enzyme horseradish peroxidase (HRP) into single identified primary afferent fibres (Brown, 1981a,b).

Fibres which enter the spinal cord via the dorsal roots all bifurcate into ascending and descending branches close to the entrance zone (Rethelyi and Szentagothai, 1973), with the exception of hair follicle afferents which only have an ascending branch in the dorsal columns (Szentagothai, 1964; Brown, 1981a,b; Patterson *et al,* 1990).

The terminations of myelinated (A) and unmyelinated (C) primary afferent fibres from the skin, differ in the superficial dorsal horn (Rethelyi, 1977, Light and Perl, 1977,1979). Light and Perl have demonstrated that the fine fibres in the lateral region of the dorsal root project predominantly to the superficial layers of the dorsal horn (Lamina I, II), while the thick fibres project predominantly to

deeper regions (Lamina II-V) of the spinal grey matter in cats and monkeys, in agreement with other studies (LaMotte, 1977).

Although the collaterals from small myelinated (Aδ) and unmyelinated (C) afferent fibres are generally associated with the superficial dorsal horn (Fitzgerald, 1984; Fyffe, 1984; Retheyli and Szentagothai, 1973), there has been some controversy over the precise distribution of fine afferent collaterals in this region. Gobel's group have reported both the degenerative changes of dorsal root afferents in lamina I and II (Gobel and Binck, 1977) and they have also observed ultrafine axons and terminals (presumed to be of C fibre origin) in lamina I of the spinal cord, following anterograde transport of HRP from cut dorsal roots (Gobel *et al,* 1980). These findings imply that there are both C and Aδ afferent fibres in lamina I. However, by using Golgi techniques, (Retheyli, 1977) and degeneration studies (LaMotte, 1977; Ralston and Ralston, 1979), it was concluded that C fibres terminate in lamina II and Aδ axons in lamina I. This is in agreement with other studies (Beal and Fox, 1976; Beal and Bicknell, 1981) who found that large axons give rise to terminals in lamina I. Studies of individual fibres (Light and Perl, 1979) and functional studies (Kumazawa and Perl, 1977) have also confirmed these results. Thus, although there may be an unmyelinated primary afferent input into lamina I, it is clear that there is a major input into this region from small myelinated Aδ fibres (Light and Perl, 1979; Fitzgerald, 1984; Fyffe, 1984).

Little experimental evidence supports a fine primary afferent input into lamina IV and V, although Light and Perl (1979) found a few Aδ collaterals in lamina V. However, many of the neurons in this lamina send dorsal dendrites superficially and thus may receive direct input from primary afferent fibres that enter more superficial laminae (Willis and Coggeshall, 1991).

Collaterals from large myelinated afferents originate from the lateral dorsal white matter and either penetrate through the medial part of the substantia gelatinosa or curve around the medial side of the dorsal horn and enter ventrally in lamina IV, where they recurve and end in large flame-shaped arbors in lamina II and III (Ramon y Cajal, 1909; Szentagothai, 1964; Scheibel and Scheibel, 1968). More recently, the superficial level to which these flame shaped arborizations reach has been a contentious issue. Degeneration studies (LaMotte, 1977) and HRP labelling (Light and Perl, 1979) demonstrate that fibres terminate only as superficial as lamina III. However, several other studies report that these arborizations reach lamina II (Proshanky and Egger, 1977; Light and Perl, 1979). It is now thought that this difference may be species dependent as it is clearly observed in monkeys (Beal and Fox, 1976) but not in cat (Brown, 1981a,b). Many

large myelinated primary afferent axons directly enter lamina IV. Scheibel and Scheibel (1968) suggest that these fibres are a separate population to those which send collaterals to the substantia gelatinosa. Szentagothai (1964) however, suggests they all come from the same population of fibres.

In conclusion, the superficial layers appear to play a specific role in nociception as they receive a major input from small diameter afferent fibres. The deeper laminae on the other hand, receive inputs mainly from non-nociceptive primary afferent fibres.

1.3.3 Classification of Dorsal Horn Neurons

The heterogenous population of dorsal horn neurons can be classified according to their responsiveness towards electrical and natural cutaneous stimuli (Iggo, 1974; Handwerker *et al,* 1975) into three broad categories.

(a) Non-Nociceptive Neurons

These cells are also known as Class 1 cells (Iggo, 1974), excited by low threshold mechanoreceptors responding to touch, hair movement and other types of innocuous tactile stimulation (Dubner and Bennet, 1983). As they reach maximal discharge frequencies below noxious stimulus intensities (see Mense, 1990), it is unlikely that they are involved in the processing of nociceptive information. They generally have small receptive fields with sharp boundaries, each low threshold mechanoreceptor responding to input from different kinds and combinations of primary afferent fibres.

(b) Multireceptive Neurons

These neurons are also known as Class II (Iggo, 1974) or wide dynamic range (WDR) cells. They have a convergent input from both Aβ low threshold mechanoreceptor afferents and small diameter (Aδ and C) nociceptive afferents (Price *et al,* 1976,1978,1979). Accordingly they have a low mechanical threshold responding to both noxious and innocuous stimulation with a higher (maximal) firing frequency upon noxious stimulation.

Multireceptive neurons are abundant in lamina V of the dorsal horn, but are also found in other lamina (Price *et al,* 1976,1978,1979; Bennet *et al,* 1981). They have large receptive fields, organised in a complex manner, with a central area responsive to both tactile and noxious stimulation surrounded by a larger area, with ill-defined borders that is responsive only to noxious stimuli. The entire excitatory receptive field may depend on anasthesia level and descending

influences (Dubner and Bennet, 1983). Many of these neurons which are prominent in lamina IV and V respond to input from sensitive, low threshold mechanoreceptors only in the intact spinal cord, but become additionally responsive to noxious stimuli when the influence of tracts descending from supraspinal sites is blocked (Brown, 1971; Handwerker *et al,* 1975).

There is considerable evidence that multireceptive neurons have an important function in pain mechanisms (see Dubner and Bennett, 1983 for a review). These neurons respond to both noxious mechanical and noxious heat stimulation with a frequency of discharge that is higher than that which can be evoked by any form of innocuous stimulation (Price *et al,* 1976,1978,1979; Hoffman *et al,* 1981). It has been suggested that the multireceptive neurons may be particularly important for discrimination of different intensities of painful stimulation (Dubner *et al,* 1989; Kenshalo *et al,* 1989). Also, human studies have showed evidence that the selective stimulation of multireceptive neurons produced pain (Mayer *et al,* 1975; Price and Dubner, 1977). In addition, many multireceptive neurons can be antidromically activated from the contralateral thalamus (Willis *et al,* 1974; Price *et al,* 1976).

(c) ***Nocispecific Neurons***

These neurons are also known as Class 3 cells (Iggo, 1974) or nociceptive specific (NS) (Price and Dubner, 1977). They respond exclusively to noxious stimulation from impulses in slowly conducting (Aδ and C) primary afferent fibres (Cervero *et al,* 1976; Menetrey *et al,* 1977). They exist abundantly in the marginal layer (Lamina 1) of the dorsal horn (Willis *et al,* 1974; Price and Mayer, 1975; Cervero *et al,* 1976; Price *et al,* 1976; Menetrey *et al,* 1977), but are also found to a lesser extent in lamina IV and V (Price *et al,* 1976;1978;1979; Menetrey *et al,* 1977; Kumazawa and Perl ,1977).

Receptive fields for nocispecific cells are usually smaller than those of WDR cells (Cervero *et al,* 1976) and sometimes show a similar sensitivity pattern of those of WDR cells (Dubner and Bennett, 1983). Two subclasses of nocispecific cells have been described, Class 3a and 3b (Cervero *et al,* 1976). Class 3a responds only to a noxious mechanical stimulus such as pinching and appears to receive input from high threshold Aδ mechanosensitive afferents (Cervero *et al,* 1976). Class 3a responds only to a noxious mechanical stimulus such as pinching and appears to receive exclusive input from high threshold Aδ mechanosensitive afferents (Cervero *et al,* 1976; Price *et al,* 1976). Class 3b cells

have input from C fibres in addition to Aδ and respond to noxious mechanical and thermal stimuli.

NS neurons do not seem to be able to detect very small changes in noxious heat above 48°C (Price and Mayer, 1975; Price *et al,* 1976). It has been suggested that NS cells are likely to be part of an ascending pathway for pain since many nociceptive specific afferents can be antidromically activated from areas anatomically associated with nociception.

Any of the cells in the latter two cell types may be important in the transmission of nociceptive information and all 3 types may have some relevance to the eventual perception of pain. Many of the 3 categories of spinal neurons mentioned above are influenced by descending tracts from various parts of the brain. Activity descending from the brainstem has been shown to preferentially inhibit nociceptive responses of dorsal horn neurons. This can be demonstrated experimentally using a reversible blockade of descending influence by local cooling of the cord (Wall, 1967; Brown, 1971; Handwerker *et al,* 1975; Cervero *et al,* 1976; Duggan *et al,* 1977). Recordings have been made from nociceptive neurons affected by tonic descending inhibition in lamina I (Cervero *et al,* 1976) and lamina IV-VI (Wall, 1967; Besson *et al,* 1975; Handwerker *et al,* 1975; Duggan *et al,* 1981). Inhibition of dorsal horn nociceptive interneurons has been demonstrated following focal electrical stimulation of various regions of the brainstem (Fields *et al,* 1977; Morton *et al,* 1983,1984; Mokha *et al,* 1985,1986; Light *et al,* 1986; Sandkhuler *et al,* 1988).

Many spinal neurons will relay information within propriospinal fibres, however, these and a significant proportion relay in ascending projections to various sites supraspinally.

1.4 ASCENDING SOMATOSENSORY PROJECTION TRACTS

After the information from skin receptors is processed in the dorsal horn it is then transmitted to supraspinal regions. A number of distinct ascending somatosensory pathways can transmit nociceptive information to supraspinal sites The spinal location of neurons comprising the major tracts are outlined below.

1.4.1 The Spinothalamic Tract

A direct projection from the spinal cord to the thalamus was first proposed by Edinger (1889), (see Willis and Coggeshall, 1991 for a review). The

locations of spinal neurons giving rise to the spinothalamic tract in rat, cat and monkey are known from investigations employing electrophysiological mapping of neurons antidromically activated from a stimulating electrode placed in the thalamus (Willis *et al,* 1974; Price *et al,* 1978; Craig and Kniffki, 1985; Palecek *et al,* 1992; Dado *et al,* 1994a,b,c), and by retrograde labelling with horseradish peroxidase (HRP) (Craig *et al,* 1989a,b; Willis *et al,* 1979) or fluorescent markers (Craig *et al,* 1989a,b).

Spinothalamic tract neurons are found throughout the spinal grey matter except for the motorneuron pools although there are concentrations of STT neurons in laminae I and V in both rat (Dado *et al,* 1994a) and monkey (Price *et al,* 1976,1979; Kumazawa and Perl, 1977; Willis *et al,* 1979). In the cat, the distribution is different.

The input to STT cells has been investigated using both electrically-evoked volleys (Foreman *et al,* 1975) and natural cutaneous stimulation (Willis *et al,* 1974; Kenshalo *et al,* 1982; Dougherty and Willis, 1992; Palecek *et al,* 1992). Many STT cells are activated by Aαβ, Aδ and C fibres in cutaneous nerves, however in some cases the input may be restricted to the smaller afferent fibre group (Foreman *et al,* 1975). The most common type of STT cell is the wide dynamic range variety (Price *et al,* 1978; Kenshalo *et al,* 1979,1982; Palecek *et al,* 1992; Dado *et al,* 1994b), though others are found including those which respond to noxious thermal stimulation of the skin (Willis *et al,* 1974; Price *et al,* 1978; Kenshalo *et al,* 1979,1982; Palecek *et al,* 1992; Dado *et al,* 1994b). Some respond to noxious cooling of the skin (Craig and Kniffki, 1985).

The STT has been regarded as a 'nociceptive pathway' for some years. This view has been supported by clinical observations in which surgical destruction (anterolateral cordotomy) of the area of the spinal cord white matter where many STT axons ascend (Willis *et al,* 1979), reduces or eliminates pain and temperature sensations below the lesion on the contralateral upper extremity (White and Sweet, 1969). STT cells may participate in the development of primary and secondary hyperalgesia. After a series of noxious heat stimuli (culminating in a mild burn), the responses of primate STT cells to further noxious heat was enhanced and the threshold decreased (Kenshalo *et al,* 1979). These changes are presumably associated with the development of primary hyperalgesia in humans. Further studies by Kenshalo *et al,* (1982) demonstrate that a mild burn also enhances the response of primate STT cells to innocuous mechanical stimuli in an area which was not subject to the burn, resembling the development of secondary hyperalgesia in humans.

Thus the evidence points to a role for the spinothalamic tract in transmission of acute and chronic nociceptive information from the spinal cord.

1.4.2 The Spinoreticular Tract

The spinoreticular tract (SRT) comprises of neurons which project from the spinal cord (mainly ascending in the ventrolateral white matter) to the brainstem reticular formation (Kevetter and Willis, 1983).

SRT neurons have been retrogradely labelled using injections of HRP into various parts of the reticular formation (Chaouch *et al,* 1983; Peschanski and Besson, 1984; Kevetter and Willis, 1983; Kevetter *et al,* 1982). Labelled cells can be seen throughout all levels of the spinal cord, generally most being contralateral to the injection site (Kevetter and Willis, 1983; Kevetter *et al,* 1982; Chaouch *et al,* 1983). In the rat, cat and monkey, most SRT neurons are located in lamina V of the dorsal horn and laminae VII and VIII of the ventral horn (Kevetter *et al,* 1982; Chaouch *et al,* 1983; Kevetter and Willis, 1983). Broadly similar results have been achieved using both True Blue (Nahin and Micevych, 1986) or Wheatgerm Agglutinin (WGA) conjugated to HRP (Peschanski and Besson, 1984).

Using retrograde transport of HRP, Lima (1990) has demonstrated a separate SRT which projects mainly ipsilaterally from lamina I and X of the spinal cord and terminates in the dorsal reticular nucleus of the medulla, which has a predominant population of nociceptive specific neurons (Villanueva *et al,* 1988).

SRT neurons are nociceptive (Fields *et al,* 1975,1977; Maunz *et al,* 1978) and thus the SRT system may play a role in processing nociceptive inputs from the spinal cord.

1.4.3 The Spinocervical Tract

The spinocervical tract (SCT) was first described in the cat by Morin in 1955. Since then, several studies have confirmed the existence of this system in cats Brown *et al,* 1976,1977,1980a,b; Cervero *et al,* 1977; Craig, 1978; Hong *et al,* 1979; Brown, 1981a,b) as well as in humans, primates, dogs, rats, mice, guinea pigs and rabbits (Truex *et al,* 1965; Mizuno *et al,* 1967; Giesler *et al,* 1979; Downie *et al,* 1988).

SCT cells project into the ipsilateral dorsolateral funiculus and terminate in the lateral cervical nucleus (Brodal and Rexed, 1953), which in turn projects to the contralateral thalamus (Craig, 1978). The location of neurons of SCT origin have been determined at all levels of the ipsilateral spinal cord (Brown *et al,*

1976,1977,1980a,b; Cervero *et al,* 1977); however, the great majority of SCT cells occupy a band across the ipsilateral dorsal horn which is centred in lamina IV (Craig, 1978; Brown *et al,* 1980a,b; Brown, 1981a).

It is generally held that the majority (70%) of SCT neurons are of the WDR type (Brown, 1971; Cervero *et al,* 1977) with receptive fields on the hairy, but not glabrous skin. Type 1 (LT) SCT neurons form the majority of the remaining 30% and the nociceptive specific units make up usually less than 5% (Brown, 1971; Cervero *et al,* 1977; Downie *et al,* 1988).

1.4.4 Postsynaptic Dorsal Columns

Using electrophysiological methods, Uddenberg (1968) first demonstrated the existence of post-synaptic fibres in the dorsal columns (PSDC). Subsequently, using both anatomical and electrophysiological methods, the PSDC has been studied further in the cat (Angaut-Petit, 1975a,b; Fields *et al,* 1977; Rustioni and Kaufman, 1977; Jankowska *et al,* 1979; Brown and Fyffe, 1981; Enevoldson and Gordon, 1984; Kamogawa and Bennett, 1986); and rat (de Pommery *et al,* 1984; Giesler *et al,* 1984; Giesler and Cliffer, 1985).

Using anatomical methods, the cells of origin of the PSDC pathway have been shown to occur predominantly in the medial part of lamina IV of the ipsilateral spinal cord (Rustioni and Kaufman, 1977; Brown and Fyffe, 1981; Enevoldson and Gordon, 1984; Giesler *et al,* 1984; de Pommery *et al,* 1984).

Many PSDC neurons (50-77%) appear to be multireceptive, being excited by the activation of both low-threshold mechanoreceptive and nociceptive primary afferents (Uddenberg, 1968; Angaut-Petit, 1975b; Jankowska *et al,* 1979; Brown and Fyffe, 1981; Brown *et al,* 1983; Kamogawa and Bennett, 1986). There are small populations of non-nociceptive and nociceptive-specific PSDC neurons also reported (Angaut-Petit, 1975b; Brown and Fyffe, 1981; Brown *et al,* 1983; Kamogawa and Bennett, 1986).

Giesler and Cliffer (1985) tested the responses of PSDC neurons to noxious heat after sensitisation to repeated noxious heating and found that 93% of the sensitised neurons tested failed to respond to noxious heat. It was concluded that these neurons do not play an important role in nociception in the rat, although similar experiments in cats yielded less clear-cut results (Kamogawa and Bennett, 1986).

1.4.5 Spinomesencephalic Tract

The spinomesencephalic tract (SMT) is a pathway from the spinal cord to the midbrain (Willis *et al,* 1979; Menetrey *et al,* 1982; Liu, 1983; Wiberg and Blomqvist, 1984; Swett *et al,* 1985). The primary targets of this pathway include the Periaqueductal Grey (PAG) intercollicular nucleus, cuneiform nucleus, pretectal nuclei and nucleus of Darkschewitson (Menetrey *et al,* 1982; Wiberg and Blomqvist, 1984; Hylden *et al,* 1986) and often with branches to the thalamus (Hylden *et al,* 1985). Retrograde transport localised the majority of SMT cells to lamina I (the marginal layer) of the dorsal horn contralateral to the injection site (Menetrey *et al,* 1982; Swett *et al,* 1985; Lima and Coimbra, 1989). However, not all investigators have demonstrated a concentration from lamina I (Willis *et al,* 1979; Liu, 1983; Wiberg and Blomqvist, 1984; Yezierski and Mendez, 1991). This difference may be attributed differences in the terminal zones accessed by tracers (Wiberg and Blomqvist, 1984; Hylden *et al,* 1985,1986; Swett *et al,* 1985; Lima and Coimbra, 1989; Yezierski and Mendez, 1991).

SMT cells can be of low threshold (LT), high threshold (HT) or wide dynamic range (WDR) type (Menetrey *et al,* 1980; Yezierski and Schwartz, 1986), with the majority of SMT cells displaying multireceptive properties (Yezierski and Schwartz, 1986).

1.5 TACHYKININS

The tachykinin family of peptides is characterised by the carboxyl terminal sequence Phe-X-Gly-Leu-Met-NH_2. They are neurotransmitter and/or neuromodulator substances in both the central and peripheral nervous systems. The family includes substance P (SP), neurokinin A (NKA), neuropeptide K (NPK), neuropeptide γ (NPγ) and neurokinin B (NKB), the amino acid sequences of which are shown in Figure 1.3.

1.5.1 Synthesis of Tachykinin Peptides

Mammalian tachykinins are the products of two distinct genes. The mRNAs that encode SP, NKA (as well as NPK and NPγ) are derived from a single gene, the PPT-1 (or PPT-A) gene (Nawa *et al,* 1984; Krause *et al,* 1987), whereas NKB is encoded by a distinct gene, the PPT-II (or PPT-B) gene (Kotani *et al,* 1986; Bonner *et al,* 1987). As shown in Figure 1.4, alternative splicing of

Figure 1.3

The mammalian tachykinin peptides.

The amino acid sequences of the five naturally occuring mammalian tachykinins, substance P (SP); neurokinin A (NKA); neuropeptide K (NPK); neuropeptide γ (NPγ) and neurokinin B (NKB).

Mammalian Tachykinin Peptides

Gene	PPT mRNA	Peptide	Sequence
SP/NKA (PPT-1)	α,β,γ	Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
	β,γ	Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	β	Neuropeptide K	Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	γ	Neuropeptide γ	Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
NKB (PPT-II)		Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2

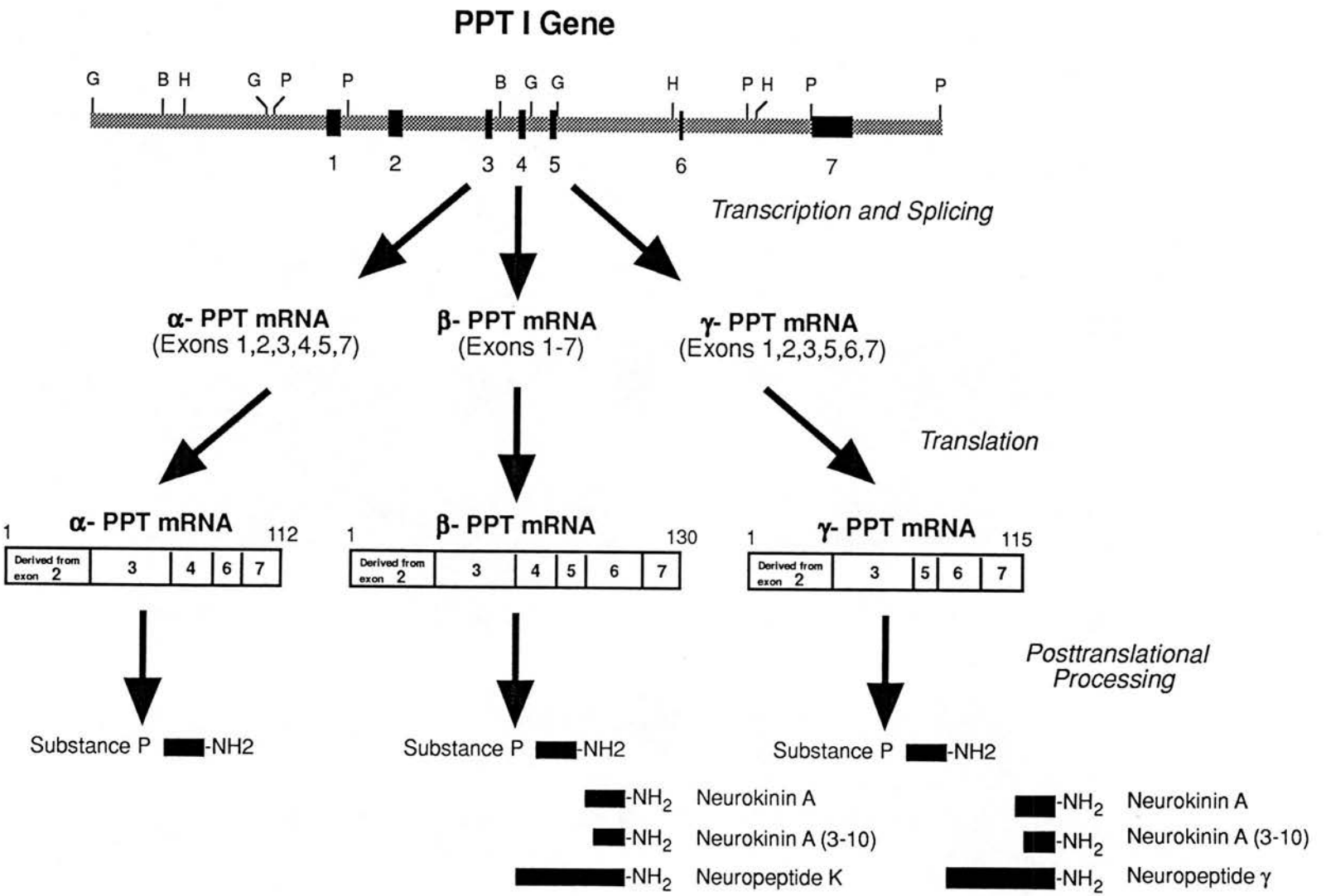
(Adapted from Helke *et al,* 1990)

Figure 1.4

Schematic diagram of the transcription and splicing of the PPT-1 gene primary transcript, and of the translation and posttranslational processing of the α-, β- and γ-PPT precursors.

The rat SP/NKA gene is depicted at the top, with the boxes denoting the exons numbered 1-7 below each. Transcription of the gene and splicing of the primary transcript yields α-, β-or γ-PPT mRNA. The mRNAs are translated with concomitant signal peptide cleavage, and the various peptides derrived from each presursor are displayed below each PPT as discussed in the text. The numbers above each PPT precursor represent the number of amino acids present in each. The abbreviations used are: G, BgIII; B, BamH1; H, HindIII; P, PstI; PPT, preprotachykinin.

(From Helke *et al,* 1990)


PPT I Gene
G B H G P P B G G H P H P P
1 2 3 4 5 6 7
Transcription and Splicing
α- PPT mRNA
(Exons 1,2,3,4,5,7)
β- PPT mRNA
(Exons 1-7)
γ- PPT mRNA
(Exons 1,2,3,5,6,7)
Translation
1 α- PPT mRNA 112
Derived from exon 2
3 4 6 7
1 β- PPT mRNA 130
Derived from exon 2
3 4 5 6 7
1 γ- PPT mRNA 115
Derived from exon 2
3 5 6 7
Posttranslational Processing
Substance P -NH2
Substance P -NH2
Substance P -NH2
-NH2 Neurokinin A
-NH2 Neurokinin A (3-10)
-NH2 Neuropeptide K
-NH2 Neurokinin A
-NH2 Neurokinin A (3-10)
-NH2 Neuropeptide γ

primary transcripts of the PPT-I gene, results in the production of four SP-encoding mRNAs differing in exon usage within the protein coding region, designated α, β , γ and δ preprotachykinin-1 mRNA (Nawa *et al,* 1984; Krause *et al,* 1987; Harmar *et al,* 1990): The β-PPT is encoded by all seven exons of the PPT-1 gene and contains sequences, which, upon post-translational processing, produce both SP and NKA: The α-PPT is missing the NKA-containing region encoded by exon 6 and thus produces SP, but not NKA : The γ-PPT is missing a 15 amino acid region encoded by exon 4 and produces both SP and NKA. The ratio between SP and NKA will vary according to differential splicing of the PPT-1 mRNA, however, SP can be derived from all three preprotachykinin mRNA transcripts, whereas NKA can only be generated from β and γ -PPT-1. NPK and NPγ are N-terminally extended derivatives of NKA that appear to be final products in the posttranslational processing of β and γ-PPT in some tissues (Arai and Emson, 1986).

In the rat, tissues expressing the PPT-1 gene generally produce on average about 80% γ-PPT mRNA and 20% of β-PPT mRNA (with slight variations in each tissue studied while α-PPT mRNA accounts for less than 1% of the PPT gene derived RNA (Carter and Krause, 1990). The relative proportion of α-, β-, and γ-PPT mRNA production is markedly species-dependent, for example, β-PPT is the predominant form expressed in human basal ganglia (Bannon *et al,* 1992), while α-PPT prevails in the bovine brain (Nawa *et al,* 1984). Thus species-specific splicing of the PPT primary transcript is observed, but within a given species, tissue-specific splicing may or may not occur. The mechanism(s) underlying alternative splicing of similar or identical transcripts in different species and tissues is unknown, however, stimulus-induced alterations in splicing could be of physiological importance.

1.5.2 Distribution of Tachykinins in the Spinal Cord and Dorsal Roots.

(ai) Distribution of Substance P

Substance P (SP) was isolated from equine brain and gut and was first detected as a peptide by Von Euler and Gaddum (1931). It was structurally characterised by Chang *et al* (1971) (see Figure 1.3) and the identity of the material was confirmed as substance P by Otsuka and Konishi (1976). The confirmed structure is Arg - Pro - Lys - Pro - Gln - Gln - Phe - Phe - Gly - Leu - Met NH_2.

Immunohistochemical studies have demonstrated that SP is found in a sub-population of dorsal root ganglion neurons (Hokfelt *et al,* 1975a,b, 1976; Salt and Hill, 1983; Leah *et al,* 1985; Ju *et al,* 1987; Merighi *et al.,* 1988; O'Brien *et al,* 1989), expressed in populations of small, dark cells, although there have also been findings in large, light cells (O'Brien *et al,* 1989). The highest levels of SP has been found in cervical and lumbar dorsal root ganglia (Smith *et al,* 1993).

Furthermore, it has been established that SP and SP-like immunoreactive material is localised in dorsal horn (Hokfelt *et al,* 1975a,1976,1977; Ljungdahl *et al,* 1978; Gibson *et al,* 1981; Charney *et al,* 1983; Ogawa *et al,* 1985; Hirakawa and Kawata, 1992) and also in a proportion of small diameter, primary sensory neurons (Hokfelt *et al,* 1975a,b; Jessell *et al,* 1979; Ju *et al,* 1987; Vedder and Otten, 1991) which have more recently been identified as A fibre high threshold mechanoreceptor units or glabrous skin C-mechanoheat units (Lawson *et al,* 1994). Surprisingly, in contrast to widely held assumptions, SP-LI was not detectable in C polymodal nociceptor units located in hairy skin (Lawson *et al,* 1994).

SP fibres are found in abundance in lamina I and II (Hokfelt *et al,* 1975a,1976,1977; Cuello *et al,* 1977; Ljungdahl *et al,* 1978; Gibson *et al,* 1981; Hunt *et al,* 1981), but they are also found in lesser abundance in lamina X, in the medial parts of laminae VI and VII and in the ventral horns of the spinal cord (Hokfelt *et al,* 1975a,1976; Gibson *et al,* 1981). Approximately 50-60% of the SP content of the dorsal horn is in primary afferent terminals and the remainder is in intrinsic spinal neurons and bulbospinal projections (Ljungdahl *et al,* 1978; Tessler *et al,* 1980), evidenced by the fact that a significant number of SP immunoreactive fibres survive both surgical and chemical deafferentations and supraspinal transections (Nagy *et al,* 1980; Hammond and Ruda, 1991). This demonstrates that some of the SP is of intrinsic origin.

As assessed by bioassay, high concentrations of SP have been demonstrated in the spinal cord (Ninkovic *et al,* 1985). This has been confirmed by showing high levels of SP in dorsal horn by radioimmunoassay (Moussaoui *et al,* 1992) and radioimmunoassay combined with high performance liquid chromatography (Ogawa *et al,* 1985; Arai and Emson, 1986). However, these are relatively crude techniques, using homogenised spinal cord and measuring basic levels of the peptide. For a more detailed study, the distribution of SP within the rat central nervous system has been studied extensively using immunohistochemistry (Hokfelt *et al,* 1975a,b,1977; Ljungdahl *et al,* 1978; Gibson *et al,* 1981; Hunt *et al,* 1981; Hirakawa and Kawata, 1992). In this

technique, antisera raised against peptides conjugated to carrier proteins, such as bovine serum albumin, are applied to tissue sections and visualised by fluorescent or other types of markers. However this technique is not ideal for two reasons, firstly, colchicine was frequently applied to raise the concentration of the peptide in the cell body to detectable levels and although the method is known to have reproducible results, Kiyama and Emson (1991) have suggested that in some cases colchicine may alter the synthesis of peptides. Secondly, the antigen may cross-react with structurally similar peptides, such as one of the neurokinins (Kanazawa *et al,* 1984), hence the careful use of the term "SP-like immunoreactivity" rather than "SP- immunoreactivity". After the sequences for rat prepro-SP mRNA became available (Chang *et al,* 1971), a more specific approach became possible with In Situ Hybridisation Histochemistry (ISHH) for localisation of cells that contain the PPT-1 mRNA (Warden and Young, 1988). Using this method PPT-1 mRNA -positive cells have been localised in neocortex, hippocampus, medial habenula, superior colliculus, central grey and dorsal horn of the spinal cord. In the dorsal horn, PPT-1 mRNA- containing cells were prominent in laminae I, II and V, dorsal root ganglia and lateral spinal nucleus.

(aii) Co-localisation of Substance P

Light microscopical studies on rat sensory ganglia have shown that many SP-containing neurons also display immunoreactivity (IR) for other peptides including calcitonin gene related peptide (CGRP) (Ju *et al,* 1987; Merighi *et al,* 1988); cholecystokinin (CCK) (Leah *et al,* 1985); galanin (GAL) (Ju *et al,* 1987); bombesin (BB) (Cameron *et al,* 1988) and neurokinin A (NKA) (Dalsgaard *et al,* 1985). Whether or not SP and somatostatin (SS)-IR co-exist, however, has been a matter of controversy. It was first reported that SS and SP exist in separate populations of sensory neurons (Hokfelt *et al,* 1976) and in agreement, radioimmunoassay techniques have shown that SP and SS have distinct distributions (Smith *et al,* 1993). However, Ju *et al* (1987) and Merighi *et al* (1988) have reported a small percentage of neurons with SP and SS co-localisation in the rat, as have Leah *et al* (1985) in the cat.

Although most studies only report the existence of single co-localisations (i.e. with only one other peptide), multiple co-localisations have been reported, where SP is co-localised with two or three other peptides (Leah *et al,* 1985; Cameron *et al,* 1988). Thus it may be possible that within a single secretory neuron, different peptides may be selectively transported to central and/or peripheral axonal branches.

SP is also known to be co-localised with 'classical' transmitters in the spinal cord. Almost all SP neurons contain enkephalin (Enk), both SP and Enk are found in neurons in lamina I and II of the dorsal horn (Senba *et al,* 1988) and are co-localised in axons and terminals of lamina I and II (Katoh *et al,* 1988). SP is also co-localised in axons and terminals of lamina I and II with 5-HT (Tashiro and Ruda, 1988) and glutamate (Glu) (DeBiasi and Rustioni, 1988).

(bi) Distribution of Neurokinin A

For many years SP was the only known mammalian tachykinin, although several tachykinins i.e. physalaemin, eledoisin and kassinin had been isolated from non-mammalian tissues (see Erspamer, 1981). However, using an antiserum with negligible crossreactivity to SP, physalaemin-like immunoreactivity was demonstrated in mammalian tissues by Lazarus and colleagues (1980). Similarly, Maggio *et al* (1983) used radioimmunoassay to provide evidence for a SP-like peptide in extracts of spinal cord and called it substance K. This peptide is now known to be neurokinin A (NKA; also known as neurokinin α or neuromedin L) and has been isolated from porcine spinal cord and sequenced (Kangawa *et al,* 1983; Kimura *et al,* 1983; Minamino *et al,* 1984a) (see Figure 1.3). The sequence is His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 which is a similar amino acid sequence to substance P at the C terminus, except it has a kassinin-like valine at position 7.

Like substance P, NKA synthesis occurs in the cell bodies of primary afferents located in the dorsal root ganglia (drg). These elements express the PPT-1 gene, thus producing SP, NKA as well as the N-terminally extended forms of NKA mentioned above (Too *et al,* 1989; Helke *et al,* 1990). Indeed, high levels of NKA-LI have been reported in the drg (Ogawa *et al,* 1985; Arai and Emson, 1986) and after intrathecal colchicine, NKA-LI has been observed in a population of predominantly small size spinal ganglion bodies. (Dalsgaard *et al,* 1985), which have been shown to incorporate radiolabelled amino acids into NKA *in vitro* (Harmar and Keen, 1984). Capsaicin treatment has been shown to reduce NKA levels in the drg by approximately 50% (Moussaoui *et al,* 1992), in agreement with Maggio and Hunter (1984), who found similar results when using an antiserum raised against kassinin. This confirms that cell bodies of capsaicin sensitive neurons, whose central terminals are located in the dorsal horn, contain NKA, in agreement with Moussaoui *et al* (1992) and Hua *et al* (1985), who reported the presence of NKA in capsaicin-sensitive sensory neurons in rats and guinea-pigs respectively.

NKA is found in high levels in the dorsal horn of the spinal cord, by the use of radioimmunoassay and high performance liquid chromatography combined with radioimmunoassay (Kanazawa *et al,* 1984; Minamino *et al,* 1984a,b; Ogawa *et al,* 1985; Shults *et al,* 1985; Arai and Emson, 1986; Tateishi *et al,* 1989; Franck *et al,* 1991). NKA has been shown to have a similar distribution to SP in the dorsal horn of the spinal cord (Ogawa *et al,* 1985), with higher amounts in lumbar than cervical and thoracic segments (Moussaoui *et al,* 1992). However, these methods only measure the overall amounts of NKA in the dorsal horn and give no information about its precise distribution within the various laminae. Dalsgaard *et al* (1985) have employed an immnohistochemical technique with a specific antiserum to NKA and detected a dense pattern of NKA-LI predominantly in the outer layers of the dorsal horn, corresponding to Rexed's laminae I and II which was markedly reduced after dorsal rhizotomy. Warden and Young (1988) have carried out in situ hybridisation to study the distribution of PPT-1 and PPT-2 in the spinal cord of the rat, with high levels of PPT-I and PPT-2 labelled cell bodies being found in laminae I and II of the dorsal horn i.e. where fine nociceptive afferents terminate.

(bii) Co-Localisation of Neurokinin A

In accordance with the existence of a common precursor for SP and NKA, there is strong evidence that NKA is co-localised with SP in the dorsal horn and dorsal root ganglia. Radioimmunoassay techniques demonstrated that SP and NKA are generally distributed in a fairly consistent manner with relatively invariant molar ratios (Ogawa *et al,* 1985; Arai and Emson, 1986) and after unilateral cervical dorsal roots had been sectioned, SP and NKA were both decreased, in a parallel fashion, in the dorsal horn. Similarly immunohistochemical studies have demonstrated that NKA-like immunoreactivity was detected in the spinal dorsal horn with a similar distribution pattern to SP, both decreasing in a parallel fashion after dorsal root transection (Dalsgaard *et al,* 1985). Using colchicine pre-treatment, these investigators have demonstrated that a population of NKA cell bodies in the dorsal root ganglia also display immunoreactivity for substance P and hence it was concluded that these peptides are co-localised in a population of primary sensory neurons. As both NKA and SP can potentially be derived from β-and γ-PPT mRNA and SP has been shown to be co-localised with many peptides and neurotransmitters, it is possible that NKA also coexists with some or all of these substances but may remain undetected.

(ci) ***Distribution of Neurokinin B.***

Neurokinin B (NKB; also known as neuromedin K or neurokinin β) was isolated from the spinal cord and then sequenced (Kangawa *et al,* 1983; Kimura *et al,* 1983; Minamino *et al,* 1984b; Nawa *et al,* 1984). The isolated sequence is Asp-Met-His-Asp-Phe-Phe-Ala-Gly-Leu-Met-NH_2 (see Figure 1.3) which is similar to the amino acid sequence of SP at the C-terminus.

NKB seems to have a different and non-overlapping distribution to NKA and SP (Kanazawa *et al,* 1984; Ogawa *et al,* 1985; Arai and Emson, 1986; Helke *et al,* 1990). Unlike the products of PPT-1, NKB is not detected in the dorsal root ganglia by radioimmunoassay (Moussaoui *et al,* 1992); ISHH (Warden and Young, 1988) or immunocytochemistry (Too and Maggio, 1992). It is also unlikely that NKB, is localised in primary afferent terminals, as after dorsal root section, there is a decrease in SP but no change in the concentration of NKB in the dorsal horn (Ogawa *et al,* 1985). Similarly, neonatal capsaicin treatment did not affect the NKB concentration in the spinal cord, while it reduced NKA and SP concentrations by approximately 50% in the dorsal horn (Moussaoui *et al,* 1992). As the content of NKB in the spinal cord was unaffected by dorsal root section, it is reasonable to suppose that NKB in the dorsal horn is localised in nerve terminals belonging to interneurons, descending or ascending fibres.

Using radioimmunoassay and high performance liquid chromatography (HPLC), NKB has been demonstrated to be at highest concentrations in the dorsal horn of the spinal cord (Ogawa *et al,* 1985; Arai and Emson, 1986; Tateishi *et al,* 1989; Moussaoui *et al,* 1992) and distributed all along the rostrocaudal axis of the spinal cord (Moussaoui *et al,* 1992). ISHH has revealed that these NKB- positive cells are present in a prominent band across lamina III, with a few cells in lamina I and II (Warden and Young, 1988). However, different results were achieved more recently when Too and Maggio (1992) carried out a double-labelling immunofluorescence study in rat spinal cord using highly specific antisera raised against SP or NKB. These investigators reported that NKB immunoreactive fibres were concentrated mainly in lamina I and II, but it was not possible to localise NKB-LI cells in the spinal cord. One possible answer to the discrepancy in the results is that although cell bodies containing NKB mRNA have been localised predominantly in lamina III (Warden and Young, 1988), perhaps these cells send fibres into the more superficial layers.

1.6 TACHYKININ RECEPTORS

There is evidence for three pharmacologically distinct mammalian tachykinin receptors (Regoli *et al,* 1987). They have been termed neurokinin NK_1, NK_2 and NK_3 receptors and although all natural tachykinins are capable of acting as full agonists at all of these 3 receptor sites (Regoli *et al,* 1987), each has its own preferred ligand (Buck *et al,* 1986 Nakanishi *et al,* 1991; Maggi *et al,* 1993).

NK_1- (SP has the highest affinity for this receptor).

NK_2- (NKA, NPK and NKγ all act preferentially at this receptor).

NK_3- (NKB is the preferred peptide).

Using molecular cloning techniques, 3 distinct genes encoding these receptors, have been isolated and characterised. A cDNA clone for the NK_2 receptor was first isolated from bovine stomach (Masu *et al,* 1987) and then also from rat stomach (Sasai and Nakanishi, 1989). Similarly, cDNA clones for NK_1 and NK_3 receptors were isolated from rat brain (Yokota *et al,* 1989; Shigemoto *et al,* 1990). All 3 tachykinin receptors are members of a superfamily of G protein-coupled rhodopsin-like receptors whose activation leads to stimulation of the phosphoinositide (PI) pathway (Helke *et al,* 1990; Maggi *et al,* 1993).

1.6.1 The Distribution of Tachykinin Receptors in the Spinal Cord

The distribution of tachykinin receptors has been investigated using pharmacological techniques, radioligand binding and autoradiography and more recently by directly investigating expression of mRNA in tissues.

(ai) Distribution of NK_1 Receptors.

By use of [^{125}I]-Bolton Hunter-Substance P ([^{125}I]-BH-SP) it has been demonstrated that NK_1 receptors are densely located in lamina I-II of the dorsal horn, intermediolateral cell column and lamina X (Ninkovic *et al,* 1985; Helke *et al,* 1986; Yashpal *et al,* 1990). This has been confirmed by measurement of mRNA for NK_1 receptors which has consistently revealed high levels of expression in the superficial spinal cord (Elde *et al,* 1990; Hershey and Krause, 1990; Tsuchida *et al,* 1990; Schafer *et al,* 1993). Using ISHH, Elde *et al* (1990) have reported high levels of NK_1 receptor expression in neurons of the dorsal horn, the area around the central canal and preganglionic autonomic neurons.

Using [^{125}I]-BH-SP in combination with rhizotomy or lesioning, it has been proposed that NK_1 receptor binding sites are located post-synaptically to

primary afferent terminals, associated with both sensory and motor neurons (Helke *et al,* 1986; Yashpal *et al,* 1991). Similar results have been achieved following neonatal capsaicin (Mantyh and Hunt, 1985; Helke *et al,* 1986).

(aii) Heterogeneity of NK_1 Receptors.

In spite of a general agreement in numerous binding studies, the introduction of highly selective non-peptide NK_1 antagonists has highlighted some important differences between species. The non-peptide NK_1 antagonist CP-96,345, has been shown to be about 100 times more potent in human, bovine and guinea-pig tissues than in those of rat or mice (Beresford *et al,* 1991; Gitter *et al,* 1991; Watling *et al,* 1991; Petitet *et al,* 1993a). The converse picture is presented by the non-peptide receptor antagonist RP 67,580, which possesses greater potency in blocking the NK-1 receptor in the rat than guinea pig (Garrett *et al,* 1991; Petitet *et al,* 1993a). These results are surprising, since the structures of rat and human receptors are very similar (95% homology) (Takeda and Krause 1991), however, using these non-peptide antagonists, NK_1 receptors can be differentiated into two broad categories.

Heterogeneity of NK_1 binding sites within the same species have also been observed on the basis of differences in binding between central and peripheral tissues (Lew *et al,* 1990; Fardin and Garret, 1991) and in the same tissue (Petitet *et al,* 1992; Kage *et al,* 1991). These investigations have all employed 'peptide' antagonists and therefore metabolic breakdown by peptidases may account for some of the observed differences. A further study employing newer non-peptide antagonists, has been carried out. Beresford *et al,* (1992), measured the potencies of CP-96,345 and GR 82334 for NK_1 receptors in preparations from guinea-pig, rabbit and rat. Both GR 82334 and CP-96,345 were substantially less potent in rat tissues compared to guinea pig preparations and rabbit aorta, also strongly suggestive of an intraspecies difference. Indeed, behavioural studies have also suggested that there are indeed two subtypes of NK_1 receptor in mouse spinal cord termed NK_{1A} and NK_{1B} (Sakurada *et al,* 1991). The mechanism for generating two NK_1 receptors from one gene has yet to be fully elucidated, however, two isoforms of the human NK_1 receptor thought to be generated through alternative splicing mechanisms have been isolated (Fong *et al,* 1992), but are they functionally different?

(bi) ***Distribution of NK_2 Receptors.***

The distribution of ligand binding to NK_2 receptor sites in the spinal cord has been studied using [^{125}I]-BH-NKA and it was found to be distinctly different from NK_1 sites. Whereas the NK_1 sites are associated with both sensory and motor neurons, the NK_2 sites are associated only with the dorsal horn, presumably the sensory afferent system (Buck *et al,* 1986) and are found in a distinct band in lamina I and II of the dorsal horn (Mantyh *et al,* 1984a; Quirion and Dam, 1985; Dam *et al,* 1990). However these results must be interpreted with caution as many sites labelled with [^{125}I] BH-NKA may have been NK_3 rather than NK_2 sites (Saffroy *et al,* 1988). Also it has been shown by Bergstrom *et al* (1987) that addition of a Bolton Hunter group can affect the receptor specificity of certain tachykinins. Finally, the NK_1 and NK_2 receptors have very similar specificity of certain tachykinin sequences (they show an overall similarity of 48%) (Yokota *et al,* 1989) and so care must be taken in interpreting ligand binding results as there may be a degree of cross-reactivity. However, when Yashpal *et al* (1990,1991) used the more selective ligands [^{125}I]-histidyl-NKA and eledoisin they also found that NK_2 receptors were present in the spinal dorsal horn. These sites are found to be present in low quantities, mostly being concentrated along the dorsal and ventromedial borders of the dorsal horn, and along the central canal of the lumbar and sacral segments. After rhizotomy studies, Yashpal *et al,* (1991) concluded that these NK_2 sites are located postsynaptically to afferent fibre terminals.

There has been some controversy as to whether NK_2 binding sites are situated in the CNS. Some investigators have reported that NK_2 sites are present only in the periphery (Saffroy *et al,* 1988; Mantyh *et al,* 1989; Sasai and Nakanishi, 1989) and using blot-hybridisation and RNAse protection analysis Tsuchida *et al* (1990) reported that no NK_2 binding sites can be found in either the periphery or the CNS (Tsuchida *et al,* 1990). However, contradicting results were achieved by both Takeda and Krause (1991) and Schafer and Weihe (unpublished observations). Using nuclease protection analysis, Takeda and Krause (1991) found low levels of NK_2 receptors in the CNS and also, Schafer and Weihe (unpublished observations) used ISHH to detect low levels of the NK_2 receptor mRNA in the spinal cord which was elevated after adjuvant-induced arthritis.

Thus, although there has been some controversy, both ligand binding (using highly selective ligands for NK_2 receptors), nuclease protection analysis and ISHH suggest that NK_2 receptors are present in the dorsal spinal cord in very low quantities.

(bii) Heterogeneity of NK_2 Receptors.

The first indication that NK_2 receptors may be heterogeneous was obtained by McKnight and co-workers (McKnight *et al,* 1988; Williams *et al,* 1988) who observed that L-659,874 potently antagonised NK_2 agonists in the rat vas deferens whilst it was weakly active in the guinea pig isolated trachea. More recent *in vitro* studies have supported the existence of NK_2 receptor heterogeneity between species recognised by different NK_2 receptor antagonists. The NK_{2A} receptor subtype is characterised by high affinity for the heptapeptide antagonists MEN 10207 and MEN 10376, and the selective NK_2 ligand MDL28564. It is preferentially expressed in rabbit pulmonary artery and bronchus, guinea pig trachea, human ileum and colon and rat spinal cord (Buck *et al,* 1990; Maggi *et al,* 1990,1991,1992; Patacchini *et al,* 1991; Wiesenfeld-Hallin *et al,* 1994). The NK_{2B} receptor subtype is preferentially recognised by the antagonists R396 and L-659,877 and is expressed in rat vas deferens and hamster trachea (Maggi *et al,* 1991,1992; Patacchini *et al,* 1991). This interspecies heterogeneity of NK_2 receptors has been supported using radioligand binding studies (Van Giersbergen *et al,* 1991).

Pharmacological and molecular binding studies have also demonstrated tachykinin receptor subtypes expressed in rat tissues (Takeda and Krause, 1991; Nimmo *et al,* 1992). Using ligand binding assays ([^{125}I]-NPγ) combined with analysis of NK_2 receptor mRNA levels by nuclease protection methods, Takeda and Krause (1991) showed that the highest level of NK_2 receptor mRNA expression was found with urinary bladder RNA and relatively high expression found with vas deferens and gastrointestinal tissue RNAs. Expression was also detected in hippocampus, striatum and spinal cord RNA preparations but the levels were about 500-fold lower than for urinary bladder. From these observations, Takeda and Krause (1991) concluded that there are two distinct NK_2 receptor types in rat tissue membranes, one type expressed in duodenum and another expressed in vas deferens and urinary bladder. However, two types of NK_2 receptor have been identified within rat urinary bladder by the displacement of [^{125}I]-NKA with the NK_2 receptor selective antagonist MEN 10207 (Nimmo *et al,* 1992). The differences observed between studies may be due to the use of different ligands, however, until two distinct receptor subtypes are molecularly characterised, these results must be interpreted with caution.

(ci) Distribution of NK_3 Receptors.

Receptor binding assays and autoradiographic studies using [^{125}I]-BH-eledoisin demonstrate that NK_3 sites are found in highest concentrations in the dorsal horn especially in its dorsal borders; while there are low concentrations in the lateral horn of the thoracic cord and around the central canal in lumbar and sacral segments (Buck *et al,* 1986; Mantyh *et al,* 1989; Yashpal *et al,* 1990,1991). Unfortunately it has been shown that this ligand can also bind to both NK_1 and NK_2 sub-types under certain assay conditions (Regoli *et al,* 1987; Quirion and Dam, 1988) and so these results do not necessarily display specific binding for NK_3 receptors. However, using a highly selective synthetic agonist ligand [^{3}H]senktide, a similar distribution of binding has been observed in the primate spinal cord (Guard and Watson, 1991), with specific binding concentrated in the superficial layers of the dorsal horn and lower levels observed throughout the dorsal and ventral horns.

(cii) Heterogeneity of NK_3 Receptors.

The study of possible NK_3 receptor subtypes has been hampered by the lack of selective agonists and antagonists, however, most recently radioligand binding studies have suggested the existence of interspecies NK_3 receptor subtypes.

Using displacement of the selective NK_3 receptor radioligand [^{125}I]-[MePhe^7]NKB with the selective NK_3 receptor agonists senktide, [MePhe^7]NKB and [Pro^7]NKB in both guinea-pig and rat cerebral cortex membranes, differences in binding can be observed. Whereas both senktide and [MePhe^7]NKB potently displace the radioactive ligand in both guinea pig and rat membranes, [Pro^7]NKB displays higher affinity for the rat compared to the guinea pig membranes (Watling *et al,* 1994). Interestingly, the non-peptide NK_2 antagonist SR 48968, whilst possessing essentially no affinity for NK_3 binding sites in the rat, is a moderately potent displacer of NK_3 binding sites in the guinea pig (Petitet *et al,* 1993b; Watling *et al,* 1994). However, as is the case for the NK_2 receptor, no information is currently available concerning the molecular determinants which underlie these apparent species variants of the NK_3 receptor and thus results must be interpreted with caution.

1.7 THE INVOLVEMENT OF TACHYKININS IN ACUTE AND SUSTAINED NOCICEPTION

The tachykinins SP and NKA are thought to play an important role in the transmission of nociceptive information in the spinal cord due to the observation that the greatest concentration of tachykinin-like immunoreactive terminals are seen in the region of the substantia gelatinosa (Ogawa *et al,* 1985), an area where fine nociceptive afferents predominantly terminate (Perl, 1984). In addition, results from various experimental observations including electrophysiological, flexor-reflex, transmitter release and behavioural studies indicate an important role for these tachykinins in both acute and sustained nociceptive pain states.

1.7.1 Acute Nociceptive States and the Possible Involvement of Tachykinins in their Production

Acute pain is that of short duration which normally occurs at the onset of noxious stimulus, lasting over 10s of seconds, and in an unanaesthetised state will evoke a withdrawal reflex from the source of injury. There are several lines of evidence indicative of a role for the tachykinins SP and NKA in the spinally-mediated transmission of acute pain.

(a) Substance P.

There is much evidence indicative of an important role for SP in transmission of nociceptive information in the spinal cord. SP was first proposed to mediate nociceptive inputs at the first sensory synapse in the spinal cord (Henry, 1976) on the basis of immunohistochemical evidence, for its presence in small diameter sensory fibres (Hokfelt *et al,* 1975a), and electrophysiological evidence for its excitation of nociceptive neurons in this region (Henry, 1976). This contention has been supported by the following :-

(ai) Transmitter Release Studies.

Both sensitive antibody microprobe and push-pull cannula perfusion techniques have been utilised to measure the release of SP-like immunoreactivity (SP-LI) in the spinal cord *in vivo* and it has been demonstrated that SP-LI is released from the spinal cord in response to noxious (but not non-noxious) mechanical and cold stimulation of the ipsilateral hindpaw (Duggan *et al,* 1987,1988; Go and Yaksh, 1987; Kuraishi *et al,* 1989; Tiseo *et al,* 1990). However, contradicting results have been obtained concerning SP release in

response to noxious thermal stimulation. Whereas Duggan *et al* (1987,1988) have demonstrated SP release in the spinal cord following a noxious thermal stimulation to the ipsilateral hindpaw, other groups of investigators (Tiseo *et al,* 1990; Kuraishi *et al,* 1989) could not demonstrate a thermally-mediated SP release even when behavioural signs were indicative of pain (Tiseo *et al,* 1990). However, the thermal stimulus used by Duggan's group was greater than the threshold for irreversible inflammatory damage, suggesting that SP is more likely to be released in a 'sensitised' state. Indeed, spinal release of SP-IR has been demonstrated following 'scalding' thermal stimulation (Go and Yaksh, 1987; Kuraishi *et al,* 1989) in addition to more prolonged inflammatory conditions (Oku *et al,* 1987; Kuraishi *et al,* 1989; Schaible *et al,* 1990; McCarson and Goldstein 1991).

Using electrical stimulation of the sciatic nerve, SP in the spinal cord has been shown to be released from small, probably unmyelinated primary afferents (Yaksh *et al,* 1980; Go and Yaksh, 1987) and the antibody microprobe detection technique has found that this release is in a discrete, focal area around the substantia gelatinosa (Duggan *et al,* 1987,1988). It should be noted that not all SP in the dorsal horn is found in primary afferent fibres, approximately 20% is thought to originate from intrinsic and descending neurons (Jessell *et al,* 1979). However, the contribution to evoked release from these seems to be minimal as in spinally-transected animals no change was observed in SP release due to noxious pinch (McCarson and Goldstein, 1991).

Thus from release studies it cannot be concluded that SP is primarily responsible for transmission of acute nociceptive responses. A certain element of tissue damage seems necessary to provoke its release to noxious thermal stimuli and so it may result in the activation of nociceptors that are not heat specific

(aii) Electrophysiological Studies.

In *in vitro,* intracellular recording studies, electrical stimulation of dorsal roots at C fibre strength or bath application of SP to both adult rat transverse spinal cord slices (Yoshimura *et al,* 1993; Urban and Randic, 1984) and neonatal hemisected spinal cord (Nagy *et al,* 1993), produces a late, slow depolarisation in dorsal horn neurons. This can be blocked by the SP antagonists Spantide (Yoshimura *et al,* 1993) and (D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11})-SP (Urban and Randic, 1984). However, the evidence that these SP-evoked responses are mediated by C-fibres is not compelling. Although the newly described NK_1 antagonist CP-96,345 specifically blocked the excitation of cat dorsal horn

neurons brought about by electrical stimulation of afferents at C-fibre strength (Nagy *et al*, 1993), in capsaicin-sensitive fibres, the antagonist CP-96,345 did not inhibit the capsaicin-evoked equivalent depolarisation. As capsaicin selectively activates a subpopulation of primary afferent fibres, the majority of which are polymodal nociceptors (Fitzgerald, 1983), these data suggest that NK_1 receptors are not involved in the activation of capsaicin-sensitive C fibres.

In numerous *in vivo* studies, SP has been microionophoretically applied to dorsal horn neurons in the spinal cord. Henry (1976) was one of the first to suggest a role for SP in pain perception when he found that ionophoretic application of this peptide to single neurons in laminae IV-VI of the cat spinal cord excited about half of the cells tested. Its failure to excite all nociceptive neurons was explained by the low rate of SP release from the micropipettes. Since then, several groups have applied SP by microiononphoresis in the vicinity of dorsal horn neurons and generally observed excitatory effects (Randic and Miletic, 1977; Sastry 1979; Zieglgansberger and Tulloch, 1979; Piercey *et al*, 1981; Kellstein *et al*, 1990; Hill *et al*, 1985; Salter and Henry, 1991). The locally ionophoresed SP can produce an excitatory effect that is slow in onset and of long duration (Henry, 1976; Zieglgansberger and Tulloch, 1979; Salter and Henry, 1991), inhibited by the selective SP antagonist CP-96,345 (Radhakrishnan and Henry, 1991) and some have found it to be selective for dorsal horn neurons that respond to noxious stimulation (Henry, 1976; Salter and Henry, 1991). However, in all these studies, heterogenous neuronal populations have been examined, which may present considerable variation in the organisation of their cutaneous afferent inputs. Furthermore, the predominant zone of fine afferent termination and also of receptors for the released transmitters, is the substantia gelatinosa (Perl, 1984), far away from most of the drug administration sites in the above studies. In some cases, complex mixed excitatory and inhibitory effects have been observed (Davies and Dray, 1980; Murase and Randic, 1984; Willcockson *et al*, 1984; Ryall and Pini, 1987) suggesting multiple sites or components of action. Also very few studies have histologically identified the neurons and this may cause a further level of variability.

There is a great deal of controversy over the role of SP in the spinal dorsal horn. Whereas it has been reported by various workers that SP selectively excites mechanically nociceptive neurons (Randic and Miletic, 1977), chemically nociceptive neurons (Wright and Roberts, 1980) or thermally nociceptive neurons (Henry, 1976; Piercey *et al*, 1980) in the dorsal horn, excitatory effects have also been seen by some workers on non-nociceptive dorsal horn neurons

(Zieglgansberger and Tulloch, 1979; Lawson *et al*, 1994). Indeed, other *in vivo* experiments indicate that some inputs to a sub-population of dorsal horn neurons are inhibited by SP, acting via an NK_1 receptor (Fleetwood-Walker *et al*, 1987,1990). Fleetwood-Walker *et al,* (1987) used a dual electrode protocol to investigate the roles of tachykinins in somatosensory processing. This involved administering drugs into the region of the substantia gelatinosa (SG) whilst recording responses of laminae IV-V spinocervical tract (SCT) neurons. Results showed that [Met-OMe^{11}]-SP (a selective NK_1 agonist) was ineffective in altering nociceptive responses, although it appeared to have an inhibitory influence over a non-nociceptive input pathway. This was confirmed in later when Fleetwood-Walker *et al,* (1990) examined effects of tachykinins on both superficial and deep dorsal horn neurons. The responses of laminae III-V dorsal horn neurons to innocuous cutaneous stimuli were attenuated by SP and the highly selective NK_1 agonist [N-acetyl-Arg^6,Sar^9,$Met(O_2)^{11}$]SP-(6-11) whereas the selective NK_1 receptor antagonists L-668,169, GR 82334 and [D-Pro^4,D-$Trp^{7,9,10}Phe^{11}$]substance P-(4-11) failed to influence neuronal responses to cutaneous pinch or noxious heat but often enhanced responses to noxious brush. However, Duggan *et al*, (1979) found that when SP was administered ionophoretically into the substantia gelatinosa of cats it failed to affect excitation of deeper spinal cord neurons elicited by non-noxious (brush) as well as noxious (heat) cutaneous stimuli. The discrepancies seen are probably due to differences in experimental procedure. In particular, differences in anaesthesia may be of relevance, as substance P-induced excitations appear to be especially sensitive to anaesthetics (Sastry, 1978).

In decerebrate, spinalised, unanaesthetised rats, intrathecal SP facilitates the nociceptive flexion reflex to both mechanical and thermal stimulation of the ipsilateral hindfoot (Wiesenfeld-Hallin, 1986; Woolf and Wiesenfeld-Hallin, 1986; Xu and Wiesenfeld-Hallin, 1992). However, the thermal stimulation is very intense (80-100°C) and is most probably damaging causing local inflammatory effects. The tachykinin antagonist spantide II selectively blocks the facilitatory effect of intrathecal SP and C-afferent conditioning stimulation on spinal cord reflex excitability without causing neural injury (Wiesenfeld-Hallin *et al,* 1990). A similar inhibition of the flexor reflex has been achieved using the highly selective non-peptide antagonists CP-96,345 (Xu *et al*, 1992a) and RP 67,580 (Laird *et al*, 1993). However, multiple sites of action inappropriate to the function of endogenous nociception such as the ventral horn (Otsuka and Yangisawa,

1980) may contribute to these results and thus this effect may not be entirely due to the action of SP.

(aiii) Behavioural Studies.

Behavioural responses provide inconsistent evidence that NK_1 receptors are involved in mediating nociceptive effects of tachykinins in the rat spinal cord. Intrathecal administration of SP or selective NK_1 agonists results in dose-dependent reciprocal hindlimb scratching, biting and licking responses directed to the caudal part of the body (Hylden and Wilcox, 1981; Piercey *et al,* 1981; Sweeney and Sawynok, 1986; Post and Folkers, 1985; Gamse and Saria, 1986; Papir-Kricheli *et al,* 1990; Laneuville *et al,* 1988; Lecci *et al,* 1991). These responses are thought to be a syndrome indicative of nociceptive stimulation and supporting this, intrathecal injection of both SP and selective NK_1 agonists has been shown to result in a dose-dependent reduction in both tail-flick latency (Picard *et al,* 1993; Cridland and Henry, 1986,1988; Yashpal *et al,* 1993; Post and Folkers, 1985) and paw-pressure latency (Sweeney and Sawynok, 1986), thus facilitating nociceptive transmission and indeed, one report claims that SP causes hyperalgesia after intrathecal administration (Akerman *et al,* 1982). However, not all studies are in agreement with SP having a primary role in nociceptive transmission, Doi and Jurna, (1981) demonstrated antinociception from 10 min after intrathecal SP injection and others have reported that SP plays no role in the behavioural responses to tail-flick and hot plate test in the mouse (Gamse and Saria, 1986) or rat (Piercey *et al,* 1981). It may be that SP is a modulator rather than a transmitter of noxious information as suggested by Sweeney and Sawynok, (1986) after they observed that pre-treatment with two doses of intrathecal SP produced complete desensitisation to the paw pressure test without altering the baseline responsiveness.

Experiments with the peptide SP antagonists have further suggested a role for SP in transmission of nociceptive input to the spinal cord. Intrathecal administration of SP antagonists prevents the aversive responses (biting, scratching and licking) induced by intrathecal SP and produces antinociception in the hot plate and Tail-flick tests (Piercey *et al,* 1981; Lembeck *et al,* 1981; Post and Folkers, 1985). However, the interpretation of these results is hampered by the fact that several peptidergic SP antagonists also possess pharmacological activities which are unrelated to blockade of SP receptors, for example vasoconstriction, (Freedman *et al,* 1988).

The more recent development of the selective non-peptide NK_1 receptor antagonists CP-96,345 (Snider *et al,* 1991), CP-99,994 (McLean *et al*, 1993) and RP 67580 (Garret *et al,* 1991) have provided new research tools with which to investigate the role of tachykinins in nociception. In adult rats, CP-96,345 has been shown to be active in both the hot plate (Lecci *et al,* 1991) and Tail-flick (Picard *et al,* 1993; Yashpal *et al,* 1993) tests and in addition, it has been demonstrated to specifically and dose-dependantly block both the behavioural responses and the attenuation of the tail-flick response induced by i.t. SP agonist (Picard *et al,* 1993). These results are at variance with those from Garces *et al,* (1992) who reported no antinociceptive activity of CP-96,345 in paw-flick and tail-flick tests or SP-mediated biting and scratching. The discrepancies between these studies may be explained by the lesser affinity of CP-96,345 for rat and mouse tissue, binding with 70-fold lower affinity to the NK_1 receptor than to that in human, gerbil and guinea-pig tissue (Beresford *et al,* 1991). It is also known to have non-specific cardiovascular depressant effects (Lembeck *et al,* 1992) and calcium channel antagonist activity (Schmidt *et al,* 1992) which contribute to the antinociceptive actions of this peptide compound (Nagahisa *et al,* 1992) and demonstrate that it is not specific for NK_1 receptors. Indeed, there was no effect on acute thermal (tail-flick and hot-plate) tests or against acetic acid induced abdominal constriction, after administration of the more recently developed selective NK_1 non-peptide antagonist CP-99,994 however in a tonic nociceptive test CP-99,994 significantly reduced the second phase of the formalin-induced paw-licking response following pre-treatment (Elliott *et al*, 1992), suggestive of a role for NK_1 receptors in a tonic nociceptive state. RP 67580 binds with high affinity to the rat NK_1 receptor (Garrett *et al,* 1991), however it has been demonstrated to be inactive in the mouse tail-flick and rat paw-pressure tests (Rupniak *et al,* 1993) and only effective in models producing a more sustained level of nociception (Rupniak *et al,* 1993), in agreement with the above study with CP,99 994.

(b) Neurokinin A

Although there is considerable evidence which suggests that SP plays a role in nociceptive transmission, it is unlikely to be the only transmitter. There may be many others, but NKA is a likely candidate for a transmitter in primary afferents. It has been found that NKA is also present in primary sensory neurons (Harmar and Keen, 1984) and is released by capsaicin from primary afferent terminals (Saria *et al,* 1986). This and the observation that by radioimmunoassay

similar distributions of NKA and SP are found in rat dorsal horn, dorsal roots and dorsal root ganglia (Ogawa *et al*, 1985), suggests that NKA and SP may be co-released from relevant afferents or may be released separately, depending on the nature of the stimulus.

Compared to SP, fewer studies have been conducted on the possible functional roles for NKA in primary sensory processing.

(bi) Transmitter Release Studies.

Studies using the antibody microprobe technique have been used to examine the release of immunoreactive NKA in barbiturate anaesthetised spinal cats following cutaneous thermal and mechanical stimulation (Duggan *et al,* 1990; Hope *et al,* 1990; Lang *et a.,* 1991). Noxious mechanical and noxious thermal stimuli both increased the levels of immunoreactive NKA in a diffuse pattern throughout the dorsal horn, which was not potentiated by tissue damage (Hope *et al,* 1990). The immunoreactive NKA persisted beyond the duration of the noxious stimulus (Duggan *et al,* 1990; Hope *et al,* 1990), taking 60 to 90 minutes to reach pre-stimulus levels, suggesting that NKA is degraded slowly when released in the spinal cord. This is a different pattern to that of SP, which is released focally into the region of the SG, but only by damaging levels of stimuli (Duggan *et al,* 1987,1988) and is degraded rapidly by peptidases (Duggan *et al,* 1992).

Thus it is evident that SP and NKA have very different release (or degradation) patterns and roles in the spinal cord. The diffuse release pattern and persistence of NKA in the spinal cord are suggestive of a role for this transmitter in prolonged spinal events and indeed its release has been observed after injection of kaolin and carrageenan into the knee joint (Hope *et al,* 1990).

(bii) Electrophysiological Studies.

There is much evidence indicative of an important role for NK_2 receptors (and NKA) in the transmission of nociceptive information in the spinal cord. These have included both *in vitro* as well as *in vivo* preparations.

After capsaicin excitation of C fibres *in vitro,* a post-synaptic depolarisation of dorsal horn neurons has been demonstrated (Urban *et al*, 1992; Nagy *et al*, 1993). The selective NK_2 antagonist MEN 10376 reversibly reduced the amplitude of this depolarisation to 30% of control (Urban *et al*, 1992). Similarly, Nagy *et al*, (1993) have demonstrated that these dorsal horn neurons which respond to capsaicin, were also excited by perfusion of both NK_1 and NK_2 agonists, which were blocked by the selective antagonists CP-96,345 and MEN

10376, respectively. However, capsaicin evoked- responses in the dorsal horn were inhibited by the selective NK_2 antagonist MEN 10376, but no change was observed after perfusion with the NK_1 receptor antagonist CP-96,345. These data suggest that the activation of dorsal horn neurons by C fibres involve mainly NK_2, rather than NK_1, receptors. Further evidence for the involvement of NKA and NK_2 receptors in capsaicin-sensitive nociceptive afferents was obtained following infusion of the selective NK_2 antagonists GR 98400 and MEN 10376 (Dougherty *et al*, 1994). Both the NK_2 antagonists reduced the responses of STT neurons to intradermal capsaicin but not to noxious or innocuous mechanical stimulation.

In anaesthetised rats (Salter and Henry, 1991) and cats (Henry and Salter, 1987), ionophoretic administration of NKA has been shown to cause excitation of both nociceptive and non-nociceptive dorsal horn neurons with a delayed, slow and prolonged excitatory effect. However, these results are at variance with those of Fleetwood-Walker *et al*, (1987,1990).

By using a dual electrode protocol in the cat, Fleetwood-Walker *et al*, (1987,1990) demonstrated that NKA caused a marked and selective facilitation of responses to noxious heat, but other activity and sensory responses (even to noxious mechanical stimuli) were unaffected. A similar effect was seen when the NK_2 agonists NKA, kassinin and [Glp^6,D-Pro^9]SP, but not the NK_1 agonists SP or spantide were applied ionophoretically into the region of the substantia gelatinosa. A facilitation of the response of SCT neurons to noxious thermal, but not mechanical nociception was found. In addition, when the NK_2 antagonists [D-Pro^4, Lys^6, D-$Trp^{7,9,10}$, Phe^{11}]SP(4-11) and [D-Tyr^4, D-$Trp^{7,9}$, Nle^{11}] SP (4-11) were ionophoresed at higher currents, both were alone able to attenuate thermal nociceptive responses.

These results were more recently confirmed in the rat using a range of recently developed agonists and antagonists, which have higher selectivity for the NK_2 receptors. Using a single electrode, extracellularly recording from rat laminae III-V neurons whilst ionophoresing drugs nearby, has shown that the highly selective NK_2 receptor antagonist L-659,874 profoundly inhibited responses to noxious heat, with no effect on the sensory responses to noxious pinch or innocuous brush. A reciprocal effect was observed after ionophoresis of NK_2 agonists, NKA or GR 64349. Thus it is concluded that although both NK_1 receptors clearly mediate excitation of dorsal horn neurons, it is not NK_1, but NK_2 receptors that are important as the physiological transducer of thermal nociceptive inputs in this model.

A role for NK_2 tachykinin receptors in the regulation of the spinal flexor reflex has also been suggested (Xu *et al*, 1991; Xu and Wiesenfeld-Hallin, 1992; Wiesenfeld-Hallin *et al*, 1994). Xu *et al*, (1991) studied the effects of intrathecal NKA, SP and MEN 10207 (a selective NK-2 receptor antagonist) on the spinal nociceptive flexor reflex. The results showed that whilst both intrathecal NKA and SP facilitate the flexor reflex in a similar manner, MEN 10207 only blocked the facilitation to NKA but not to SP consistent with a role for NK-2 receptors in the mediation of spinal reflex excitability. This group followed with an investigation of the effect of intrathecal NKA on spinal reflex excitability in response to cutaneous thermal and mechanical stimulation (Xu and Wiesenfeld-Hallin, 1992). Both thermal and mechanical stimulation were substantially facilitated by NKA, the thermal with a much greater facilitation response than mechanical (although the thermal stimulus was at a damaging temperature of 80°C).

(biii) Behavioural Studies.

Although far fewer behavioural studies have been carried out for NKA than for SP, evidence is accumulating to suggest that NK_2 receptors are extremely important in mediating nociceptive transmission in the spinal cord. Intrathecal injection of NKA or a selective NK_2 agonist caused similar reciprocal hindlimb biting, licking and scratching responses to those described for SP (Gamse and Saria, 1986) in addition to dose-dependent, transient decreases in the tail-flick and paw-flick response latencies to a noxious thermal stimulus (Cridland and Henry, 1986; Laneuville *et al,* 1988; Fleetwood-Walker *et al,* 1990; Picard *et al,* 1993). The inverse effect was seen with intrathecal injection of the NK_2 antagonist [D-Pro^4, Lys^6, D-$Trp^{7,9,10}$, Phe^{11}]$SP(4\text{-}11)^4$ (D-Pro^4) (Fleetwood-Walker *et al,* 1990), with an increased response latency in Tail-flick and hot-plate tests.

A more recent study examined the non-peptide NK_2 antagonist SR 48968 as an inhibitor of the reaction time in the rat tail-flick and also on the decrease of reaction time induced by the intrathecal injection of the selective NK_2 agonist NKA-(4-10) (Picard *et al,* 1993). The results show that there was a potent decrease in tail-flick reaction time after administration of NKA-(4-10), which persisted for an extended period of time (26 minutes) and was selectively blocked by SR 48968, suggesting a role for NKA in transmission of prolonged facilitation of nociception.

1.7.2 Sustained Nociceptive States and the Possible Involvement of Tachykinins in their Production.

Persistent pain and hyperalgesia occurs following peripheral tissue injury which produces changes in the function of the nervous system. The hypersensitivity which results as a consequence of tissue injury and inflammation is due to a considerable extent of increased sensitivity of primary afferent nociceptors that innervate the area of peripheral damage (Bessou and Perl, 1969; LaMotte *et al*, 1992). This increased responsiveness in primary afferents, due to an injuring stimulus in their receptive field area, may not affect responses in central neurons (Treede *et al*, 1992). However, additional mechanisms can lead to increased responses in the neurons within the spinal cord (central sensitisation: Woolf, 1983; Treede *et al*, 1992) which may also contribute to the phenomenon of a 'sustained' nociceptive state. The induction of central sensitisation is critically dependent upon the activation of nociceptive afferent fibre inputs (Wall and Woolf, 1984; Woolf and Wall, 1986) and has been observed following brief electrical stimulation of high-threshold afferent fibres (Woolf and King; 1990), the application of chemical irritants to skin (Dougherty and Willis, 1992; LaMotte *et al*, 1992) and following peripheral inflammation (Schaible *et al*, 1990). There are several lines of evidence indicative of a role for the tachykinins SP and NKA in the spinally-mediated transmission of sustained nociception.

(a) Substance P.

(ai) Biosynthesis and Transmitter Release Studies.

Inflammatory models of formalin and CFA have been shown to increase the biosynthesis of SP-IR and mRNA encoded by the preprotachykinin (PPT)-1 gene in the spinal dorsal horn and dorsal root ganglia of rats (Minami *et al*, 1989; Donaldson *et al*, 1992; Noguchi and Ruda, 1992; McCarson and Krause, 1994).

Following bilateral injection of adjuvant into the rat hindpaws the levels of PPT mRNA were significantly increased in both the dorsal root ganglia at L_4-L_6 levels and also in the lumbar spinal cord (Minami *et al*, 1989). Similarly, after unilateral injection of adjuvant or formalin, increase in PPT mRNA level was observed only on the treated side of the spinal cord (Minami *et al*, 1989; Noguchi and Ruda, 1992) and dorsal root ganglia (Minami *et al*, 1989; Donaldson *et al*, 1992; McCarson and Krause, 1994). Using ISHH, Noguchi and Ruda (1992) have demonstrated in laminae I, II and V/VI ipsilateral to the inflammation there was a differential increase in the number of neurons exhibiting PPT mRNA. In

lamina I there was a greater than 200% increase in the number of spinal projection neurons containing PPT mRNA and using fluorogold labelling 61% of these were shown to be SMT neurons. It is possible that not only afferent, but also spinal (local) SP neurons play a role in sustained nociception.

In addition to an increased biosynthesis of transmitter, an increase in the NK_1 receptor gene expression and NK_1 binding sites have been observed after induction of inflammation by formalin and CFA (Schafer *et al*, 1993; Stucky *et al,* 1993; McCarson and Krause, 1994). Four and eight days after unilateral CFA-induced inflammation in rats, Stucky *et al* (1993) reported increases in BH-[^{125}I]SP binding in laminae I and II of the ipsilateral spinal cord, in agreement with Schafer *et al* (1993) who used ISHH to detect an almost 200% increase in NK_1 receptor gene expression within small to medium sized laminae I-II neurons 6 days after CFA injection. Similarly, using solution hybridisation-nuclease protection techniques an almost two fold increase in NK_1 receptor mRNA was detected in the rat lumbar dorsal horn 4 days after CFA or 2-4 hours after formalin injection (McCarson and Krause, 1994).

Inflammatory and 'sensitising' models have also been demonstrated to increase the release of SP in the dorsal horn. Spinal release of SP immunoreactivity (SP-IR) has been demonstrated following a 'sensitising' thermal stimulation (Go and Yaksh, 1987; Kuraishi *et al,* 1989) in addition to more prolonged inflammatory conditions (Oku *et al,* 1987; Kuraishi *et al,* 1989; Schaible *et al,* 1990; McCarson and Goldstein, 1991). *In vitro* immunochemical studies have revealed that after intraplantar injection of formalin into the rat hindpaw, there is a resulting increase in the release of SP-like immunoreactivity (SP-LI) in the ipsilateral spinal cord (Kuraishi *et al*, 1989; McCarson and Goldstein, 1989,1991). A biphasic release was observed by McCarson and Goldstein (1991) with early (0-20 min) and late (20-60 min) increases in SP-LI consistent with observations of two corresponding phases of nociceptive hyperalgesic behaviour (Chapman and Dickenson, 1993).

Carrageenan- or carrageenan/kaolin- induced inflammation takes several hours to develop and represents a useful tool to study acute inflammatory pain states. Three hours after bilateral carrageenan injection, a significant increase in both the spontaneous and capsaicin-evoked release of SP-LI has been detected in the superfusates collected from the dorsal horn (Garry and Hargreaves, 1992), indicating that an enhanced release of SP occurs in the dorsal during inflammation. Similarly, release of SP in the dorsal horn occurred following

innocuous mechanical stimulation of a carrageenan/kaolin-inflamed (Schaible *et al*, 1990) or a CFA-inflamed (Oku *et al*, 1987) but not a normal knee joint.

Collectively these results suggest that biosynthesis of SP in the spinal cord and primary sensory neurons and NK_1 receptors in the dorsal horn can be increased by a sustained and inflammatory pain state.

(aii) Electrophysiological Studies.

When C-fibres are repetitively stimulated at high frequencies, the phenomenon of 'wind-up', a progressive increase in the number of action potentials evoked per stimulus, occurs in dorsal horn neurons (Mendell, 1966). In addition to causing wind-up, brief repetitive stimulation of C-afferents also induces prolonged increase of spinal cord excitability (Wall and Woolf, 1984). Substance P may be involved to some extent in wind-up as it has been shown that ionophoresis of SP enhances the response of dorsal horn neurons to C-fibre input (Zieglzansberger and Tulloch, 1979; Kellstein *et al*, 1990), and causes a long-lasting slow depolarisation (Murase and Randic, 1984) which resembles the slow prolonged depolarisation produced by repetitive stimulation of C afferent fibres. This is strengthened by the observation that SP enhanced, whilst the SP antagonist [D-Pro^2,D-Trp^{7,9}]-SP (DPDT) attenuated 'wind-up' caused by repeated simulation of C, but not A afferents (Kellstein *et al*, 1990) and further that responses to electrical stimulation of C afferents and 'wind-up' of the flexor reflex were blocked the NK_1 antagonist RP 67,580 in a dose-dependent manner without affecting the baseline reflex (Laird *et al,* 1993). This is also consistent with the results of M^{c}Neill *et al* (1989), who observed that administration of capsaicin to rats at birth caused a depletion of IR-SP in the dorsal horn and a corresponding reduction in the number of cells exhibiting 'wind-up'. The exact mechanism by which SP produces the slow depolarisation in dorsal horn neurons has yet to be elucidated; however there has been a report which suggests that SP produces a prolonged (up to 4 mins) increase in intracellular calcium release from intracellular stores (Womack *et al*, 1988).

A spinal role for NK_1 receptors in the response of dorsal horn nociceptive neurons to peripheral inflammation has also been demonstrated, using the formalin model (Chapman and Dickenson, 1993). The selective non-peptide NK_1 receptor antagonist RP 67,580 had mimimal effects on the response to acute C-fibre stimulation, however using the same dose, RP 67580 produced marked and dose-dependent inhibitions of both phases of the formalin response of dorsal horn neurons which could be clearly separated from vehicle effects (Chapman and

Dickenson, 1993). However, it should be noted that RP 67580 was dissolved in a very acidic solution (10mM HCl) which would be likely to produce damaging effects.

Recently, a novel model of peripheral injury induced by UV irradiation has been utilised (Thompson *et al*, 1994). Behavioural hyperalgesia (both thermal and mechanical) was induced in neonatal rat pups 1-3 days following UV irradiation of one hind-paw and the spinal cord was subsequently removed, hemisected and maintained in vitro at 4°C. A and C-fibre evoked ventral root potential (VRP) responses were examined in these isolated *in vitro* spinal cord preparations and compared with those in naive untreated animals. Following UV irradiation, it was observed that both A- and C- fibre evoked responses were enhanced. The contribution of NK_1 receptors was then assessed using bath perfusion of the selective antagonists CP-96,345 and RP 67580 and it was observed that although the prolonged C-fibre evoked VRPs were not affected by NK_1 antagonists under normal conditions, the enhanced A- and C- evoked VRPs following UV injury showed a significant NK_1 component that was associated with behavioural hyperalgesia. These results suggest that NK_1 receptors may contribute to the phenomenon of 'central sensitisation' and relate to the behavioural hypersensitivity observed.

The excitability of spinal flexor reflexes in experimental animals has been shown to be increased by injury or inflammation in the periphery (Woolf, 1983; Ferrell *et al*, 1988). Similar, though more transient increases in flexor reflex excitability can be induced by high intensity electrical conditioning stimuli to C afferent (but not Aδ or Aβ afferent) fibres (Wall and Woolf, 1984; Woolf and Wiesenfeld-Hallin, 1986). This effect may be analogous to the 'wind-up' seen in dorsal horn cells after repeated C-fibre afferent stimulation to their receptive fields (Mendell and Wall, 1965). Pre-treatment of the sciatic nerve with capsaicin abolishes this facilitation (Woolf and Wall, 1986), suggestive of a role of neuropeptides. Indeed, intrathecal administration of SP is shown to mimic the electrical conditioning stimuli by facilitating the nociceptive flexor reflex for 4-5 minutes (Woolf and Wiesenfeld-Hallin; 1986; Wiesenfeld-Hallin *et al*, 1990; Xu *et al*, 1992). Intrathecal pre-treatment with the selective NK_1 antagonists spantide II (Wiesenfeld-Hallin *et al*, 1990), CP-96,345 (Xu *et al*, 1992) and RP 67,580 (Laird *et al*, 1993) effectively antagonise the facilitatory effect of SP on the flexor reflex. The non-peptide antagonist CP-96,345, at effective doses, not only attenuated facilitation evoked by the electrical conditioning stimulus, but also depressed the reflex response such that the conditioned response was considerably

smaller that the baseline response seen immediately before the conditioning response (Xu *et al,* 1992). As CP-96,345 is active at calcium channels (Schmidt *et al,* 1992), non-specific activity at these channels may account for the differences observed on the baseline reflex and thus suggests that the facilitation of the spinal flexor reflex is mediated NK_1 receptors whilst the baseline reflex is not.

(aiii) Behavioural Studies.

Many different models are used to simulate sustained or chronic pain associated with inflammation without major tissue damage. Immersion of the rat's distal tail region in hot water results in a sustained noxious cutaneous stimulation characterised by a reduction in tail-flick reaction time which both peptide and non-peptide NK_1 antagonists have been shown to dose-dependently increase (Cridland and Henry, 1988; Yashpal *et al,* 1993) suggesting a role for SP in a sensitised model of pain.

Intraplantar injection of formalin is associated with a characteristic biphasic licking and biting behaviour pattern. Early SP antagonists only inhibited the first phase of the formalin response (Ohkubo *et al,* 1990), but this could have resulted from degradation of these drugs prior to the second phase of the formalin response. More recently, non-peptide NK_1 antagonists have been used. CP-96,345 inhibited the late phase of the response in a dose dependent manner (Yamamoto and Yaksh, 1991; Birch *et al,* 1992; Nagahisa *et al,* 1992; Yashpal *et al,* 1993). Yashpal *et al,* (1993) demonstrated stereospecificity of CP-96,345 as its inactive isomer CP 96,344 was ineffective in the formalin test. However, others have found that CP 96,344 attenuated the second phase of the formalin-induced paw-licking response with a potency equal to its active isomer (Nagahisa *et al,* 1992). RP 67,580 binds with higher affinity to rat tissues than CP-96,345 but again there are discrepancies in the formalin tests between groups. Whereas some groups (Chapman and Dickenson, 1993; Garrett *et al,* 1991) have shown RP 67580 produces marked inhibitions in both phases of formalin response in both mice and rats, Rupniak *et al,* (1993) have shown that it inhibited both phases in mice and gerbils, but not rats and guinea pigs. This is a peculiar result as RP 67580 was able to attenuate the response to formalin in gerbils, but not guinea-pigs, both species bearing the human-type NK_1 receptor as defined using CP 96345 (Rupniak *et al,* 1993). These authors have shown evidence for calcium channel antagonist activity of RP 67580 *in vitro* and thus the discrepancy may not be attributable to a specific interaction with NK_1 receptors but mediated in significant part by calcium channel blockade.

Intraplantar injection of carrageenan is associated with a rapid oedematous increase in paw volume and a reduction in nociceptive pressure threshold 3-5 hours after the injection. Although the racemic mixture of CP-96,345 has been reported to show antinociceptive and anti-oedema activity in the carrageenan-induced hyperalgesia in the rat (Birch *et al,* 1992) the inactive isomer CP 96,344 can attenuate the carrageenan-induced paw oedema and hyperalgesia with a potency equal to its active isomer (Nagahisa *et al,* 1992). This suggests that SP may not be a mediator in this inflammatory model or that Ca^{2+} channels play an overriding role. However it has been shown that CP-96,345 stereospecifically inhibited mustard oil-induced rat foot oedema in rats and acetic acid induced writhing in mice (Lembeck *et al,* 1992; Nagahisai *et al,* 1992) suggesting a role for NK_1 receptors in these models of pain.

In more long-term pain states, such as caused by sciatic nerve constriction injury in a model of neuropathic pain (Yamamoto and Yaksh, 1992) or CFA (Hylden *et al,* 1992), thermal hyperalgesia was unaffected by capsaicin treatment given two days after sciatic nerve constriction, despite a significant depletion of SP-IR in the dorsal horn in both of these studies. However, neonatal administration of capsaicin was able to prevent the hyperalgesia produced in a model of neuropathic pain (Meller *et al,* 1992) suggesting that neuropathic pain state is mediated by capsaicin-sensitive afferents. This does not necessarily imply a role for SP as capsaicin-sensitive afferents may contain other putative transmitters. Also, Basbaum *et al,* (1991) have shown that after sciatic nerve constriction there is a dramatic loss of large myelinated fibres with a smaller but significant reduction in the small unmyelinated population and as capsaicin is known to destroy mainly small, unmyelinated fibres there is still an element of uncertainty.

(b) Neurokinin A.

(bi) Biosynthesis and Transmitter Release Studies.

As adjuvant or formalin induced inflammation and hyperalgesia leads to increased biosynthesis of SP-IR and mRNA encoded by the PPT-1 gene (Minami *et al*, 1989; Donaldson *et al*, 1992; Noguchi and Ruda, 1992, McCarson and Krause, 1994), then there is also the potential for NKA to respond similarly. However, there is as yet no direct evidence for an increase in NKA or NK_2 receptor biosynthesis due to inflammation and hyperalgesia. Nonetheless,

increased release of NKA has been observed in the spinal cord after injection of kaolin and carrageenan into the knee joint (Hope *et al,* 1990).

(bii) Electrophysiological Studies.

Although there is evidence that SP is involved in wind-up (Kellstein *et al,* 1990), as yet there is no evidence for the involvement of NKA in wind-up. However, since both SP and NKA can be produced together (Nawa *et al,* 1984) then it is possible either SP or NKA or both are involved in 'wind-up'. Indeed, in the *in vitro*, hemisected spinal cord model (Thompson *et al,* 1994) both the amplitude and area of prolonged C fibre-evoked ventral root responses (VRP), (which underlie the phenomenon of wind-up) were demonstrated to be significantly reduced by the selective NK_2 antagonist MEN 10376 but not the NK_1 receptor antagonists CP-96,345 or RP 67580 in naive animals. However, following peripheral hyperalgesia induced by UV irradiation, the enhanced, prolonged VRP evoked by C-fibre stimulation was significantly reduced by both NK_1 and NK_2 receptor antagonists (Thompson *et al,* 1994) demonstrating a role for NK_1 receptors in the facilitatory effect produced.

In decerebrate, spinalized, unanaesthetised rats, stimulation of both cutaneous afferent fibres in the sural nerve and muscle afferent fibres in the gastrocnemius-soleus nerve at C fibre intensity will elicit a flexor reflex response in posterior biceps femoris/semitendinosus muscles (Wall and Woolf, 1984; Xu *et al*, 1991). Administration of a conditioning stimulus (CS) (20s at 1 Hz) to the gastrocnemius nerve at an intensity that activates unmyelinated axons produces a marked increase in the excitability of the reflex, peaking at 20-30 minutes and lasting for up to 100 minutes (Wall and Woolf, 1984; Xu *et al*, 1991). Pre-treatment with the selective NK_2 antagonist [Tyr^5, D-Trp,$^{6,8,9}Arg^{10}$] neurokinin A-(4-10) (MEN 10207) (Maggi *et al,* 1990) effectively blocked the long-term reflex facilitation to the gastrocnemius nerve stimulation (Xu *et al,* 1991). The facilitation of the flexor reflex was totally abolished with no recovery for up to two hours suggesting a potent role for NK_2 receptors in the facilitation of the flexor reflex response.

(biii) Behavioural Studies.

As yet there are no reports of the behavioural responses mediated by NK_2 receptors in sustained nociceptive states.

1.8 BIOCHEMICAL CHANGES IN SPINAL CORD NEURONS DURING SUSTAINED NOCICEPTIVE INPUT.

As outlined above, a role for tachykinins in contributing to a sustained nociceptive state has been suggested, however, the intracellular mechanisms by which tachykinins might bring about a sensitisation of dorsal horn neurons are not clear. It is possible that tachykinins trigger alterations in membrane excitability through interactions with second messenger systems and protein kinases which phosphorylate membrane bound proteins (Nestler and Greengard, 1983).

Phospholipase (PLC) is an enzyme which catalyses the hydrolysis of polyphosphatidylinositol into the intracellular messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). Following its production, IP_3 stimulates the release of Ca^{2+} from internal stores; on the other hand DAG stimulates the translocation and activation of protein kinase C (PKC). When activated by DAG, PKC phosphorylates specific substrate proteins that contribute to various cellular processes, including neurotransmitter release and transduction (Nishizuka, 1986).

Noxious stimulus-induced sensitisation of dorsal horn neurons may depend, in part, on the PLC second messenger system. Consistent with a role in sensitisation of dorsal horn neurons, stimulation of PKC with phorbol esters and synthetic DAG, or the intracellular microinjection of PKC has been found to enhance Ca^{2+} currents (De Riemer *et al,* 1985) which may increase neuronal excitability as well as presynaptic transmitter release and to reduce both Ca^{2+}-dependent K^+ currents (Alkon *et al,* 1986) and Cl^- currents (Madison *et al,* 1986) and thereby result in the prolongation of depolarisation and afterdischarges associated with an inhibition of spike number.

Indeed, it has recently been suggested that PKC might play a role in excitation of polymodal nociceptors as activation of PKC leads to the depolarisation of the C-fibres of the rat vagus (Rang and Ritchie, 1988) and excitation of the capsaicin-sensitive C-fibres in the tail of the neonatal rat spinal cord and tail preparation (Bettaney *et al,* 1988; Dray *et al,* 1988). Supporting this, the nociceptive behaviour in rats following intraplantar injection of formalin was promoted by intrathecal administration of a phorbol ester or SC-10 which stimulate PKC and reduced by a non-selective kinase inhibitor H7 (Coderre, 1992; Coderre *et al,* 1993; Yashpal and Coderre, 1993) or the selective PKC inhibitor, chelerythrine (Coderre *et al,* 1994). In addition, after application of bradykinin (a potent pain-producing peptide that stimulates sensory afferent fibres including polymodal nociceptors (Erdos, 1979)) to the neonatal rat dorsal root ganglion

neurons, the resulting electrophysiological response, as well as an associated increase in $^{45}Ca^{2+}$ uptake, could be mimicked by phorbol esters and inhibited both by a down-regulation of PKC and by staurosporine (a PKC inhibitor) (Burgess *et al,* 1989).

The involvement of PKC in long-term central changes has been implicated by the use of models of peripheral mononeuropathy (Hayes *et al,* 1992; Mao *et al,* 1993) and monoarthritis induced by Complete Freund's Adjuvant (Tolle *et al,* 1994). In the peripheral mononeuropathy model designed by Bennett and Xie, (1988), Hayes *et al,* (1992) have demonstrated that monosialoganglioside (which, they report can inhibit the translocation of PKC) reduces behavioural hyperalgesia associated with the chronic constriction injury (CCI). More recently, Mao *et al,* (1993) used [^{3}H]PDBu to examine the three dimensional spatial patterns of changes in membrane bound PKC in the lumbar spinal cords of rats with peripheral mononeuropathy. Consistent with behavioural thermal hyperalgesia, CCI rats examined 3 or 10 days after sciatic nerve ligation produced a 3D pattern of increased membrane bound PKC on the ipsilateral lumbar spinal cord, mainly within spinal cord laminae I-IV and V-VI of lumbar segments L_2-L_5. On the contralateral lumbar spinal cord a much smaller increase was also associated with laminae I-IV and V-VI of lumbar segments L_2-L_5. After treatment with GM1 ganglioside (a glycosphingolipid which was reported to prevent PKC translocation/activation) both the 3D increases in spinal cord membrane-bound PKC and nociceptive behaviours were reliably reduced. Using a model of experimental monoarthritis, Tolle *et al,* (1994) revealed a bilateral increase of [20-^{3}H(N)]-phorbol,12,13-dibutyrate ([^{3}H]PDBu) binding and an expansion of [3H]PDBu binding to deeper laminae of the dorsal horn following unilateral inflammation. Whether the increased binding of [^{3}H]PDBu resembles de novo target-gene derived synthesis of PKC or functional activation of present but inactive forms of PKC is yet to be elucidated.

PKC involvement has also been implicated in another model of synaptic enhancement. Long term potentiation (LTP), a stable form of long-lasting synaptic plasticity, evoked by brief high-frequency stimulation (Bliss and Lomo, 1973). There is a large body of evidence that PKC has a critical role in the mediation of LTP (Akers *et al,* 1986; Reymann *et al,* 1988; Malinow *et al,* 1989; Huang *et al,* 1992; Wang and Feng, 1992; Meberg *et al,* 1993). Akers and co-workers (1986) proposed that PKC is translocated from the cytosol to the cell membrane after LTP induction and persistently activates substrate proteins to maintain LTP (Akers *et al,* 1986). Consistent with this hypothesis, extracellular

application of PKC inhibitors during LTP induction blocks LTP maintenance, leaving the induction relatively unaffected (Reyman *et al,* 1988). Also, Meberg *et al,* (1993) measured mRNA levels of PKC subtypes 3 days after induction of LTP (long term enhancement of synaptic transmission) in the hippocampus. Altered mRNA levels correlated significantly with alterations in synaptic efficacy, rats with greater synaptic enhancement having a lowered gene expression of both β-PKC and γ-PKC but not α-PKC. Other data suggest that PKC is involved in the induction of LTP, as intracellular injection of PKC inhibitors into CA1 pyramidal cells reduced the initial LTP magnitude and shortened the duration (Malinow *et al,* 1989; Huang *et al,* 1992; Wang and Feng, 1992). Using intracellular administration of the specific PKC inhibitor peptide PKC (19-31), the initial LTP was reduced to about half the normal size (Malinow *et al,* 1988; Wang and Feng, 1992), indicating a requirement of PKC for the induction phase of LTP.

A role for NK_1 and NK_2 receptor agonists in PKC-induced sustained neuronal activation is quite possible since both NK_1 and NK_2 receptors appear to act largely through phosphoinositide hydrolysis (Hanley *et al,* 1980; Bristow *et al,* 1987). Rusin *et al,* (1992,1993) have investigated this possibility by examining the effects of tachykinins and phorbol esters on cytosolic calcium concentration in acutely isolated neurons from the dorsal horn of the spinal cord. They found that PDBu (a phorbol ester PKC activator) and forskolin (an adenylate cyclase activator) applied for 4 minutes, mimicked the effects of tachykinins by producing a marked and prolonged potentiation of the NMDA-induced current responses of the isolated dorsal horn neurons. Staurosporine (an agent known to inhibit both protein kinase C and cAMP-dependent protein kinase) reduced the tachykinin evoked potentiation of the NMDA response. These results have been further substantiated by the unpublished observations from Carter and Krause that phorbol ester can activate transcription of the SP/NKA gene (Helke *et al,* 1990). In addition, expression of a cloned cDNA encoding the human NK_2 receptor in CHO cells, mediates powerful NKA-stimulated arachadonic acid (AA) liberation required influx of extracellular Ca^{2+} in addition to a concomitant PKC activation (Arkinstall *et al,* 1994). The PKC species involved was identified as the PKCα isoform by employing specific antibodies for immunoblot analysis. Preincubation with pertussis toxin had no effect on Ca^{2+} mobilisation but, it partially inhibited AA release and completely blocked PKCα translocation, demonstrating that NK_2 receptor activation may regulate two distinct but converging signal transduction pathways regulated by different G protein species and involving Ca^{2+} influx and PKCα activation.

Interestingly, the increased expression of preprodynorphin mRNA in ipsilateral superficial dorsal horn elicited by intraplantar carrageenan, is blocked by NK_2 receptor antagonists (Parker *et al,* 1993), again consistent with a role of a signal resulting from phosphoinositide hydrolysis such as PKC activation.

1.9 MOLECULAR MECHANISMS OF NEURONAL PLASTICITY INDUCED BY SYNAPTIC INPUT

Increases in intracellular Ca^{2+} and the activation of PKC result in the increased expression of immediate early genes such as c-fos (Morgan and Curran, 1986), whose transcription is activated rapidly and transiently within minutes of stimulation. The expression of late response genes is induced more slowly (Merlie *et al,* 1984), the products of which are thought to serve more specific effector functions in the neuronal response. The protein products of immediate early genes (e.g. c-fos) act as third messengers that are believed to be involved in the transcriptional control of genes that encode a variety of neuropeptides including tachykinins.

1.9.1 Noxious Stimulus-Induced Expression of c-fos.

Noxious stimulation leads to the expression of immediate early genes (IEGs) and their protein products. C-fos can be rapidly induced in rat spinal neurons by brief primary afferent stimulation (Hunt *et al,* 1987; Bullit, 1989; Leah *et al,* 1992). After activation of small diameter cutaneous sensory afferents with a brief noxious heat (Hunt *et al,* 1987) or noxious mechanical pinch (Bullit, 1989; Leah *et al,* 1992) there was a rapid appearance (within 30-120 minutes) of the c-fos protein-like immunoreactivity (Hunt *et al,* 1987; Bullit, 1989; Leah *et al,* 1992) in both superficial (laminae I-II) and deeper (III-VII) laminae of the appropriate ipsilateral lumbar dorsal horn segment.

Local treatment with mustard oil, formalin or carrageenan provide experimental models of short term hyperalgesia and in general have also been found to generate a very rapid increase in the levels of the protein product of the c-fos proto-oncogene (Hunt *et al,* 1987; Draisci and Iadarola, 1989; Presley *et al,* 1990; Williams *et al,* 1990; Pretel and Piekut, 1991; Noguchi *et al,* 1991,1992; Leah *et al,* 1992). Both cutaneous application and intramuscular injection of the chemical irritant mustard oil (known to selectively activate C fibres) resulted in the rapid appearance of c-fos protein -LI in nuclei of postsynaptic dorsal horn neurons

of adult (Hunt *et al,* 1987; Pretel and Piekut, 1991), but not neonatal rats (Williams *et al,* 1990), suggesting that mustard oil can stimulate small diameter fibres in the rat only when there is mature functional connectivity in the spinal cord. In the adult, 2 hours after cutaneous application of mustard oil to the hindlimb, c-fos-LI was localised to nuclei predominantly in laminae I and II of lumbar segments with only a few labelled neurons in the deeper layers; in agreement with the distribution of nociceptive primary afferents (Light and Perl, 1979). However, injection of 5% mustard oil into the medial gastrocnemius muscle evoked c-fos-LI in nuclei largely in the marginal layer I and layers IV-VII of lumbar segments 4-5 (Hunt *et al,* 1987). Again this expression is consistent with the somatotopic representation of this muscle in the dorsal horn and with major terminations of small diameter nociceptive afferents of muscle in layer I rather than layer II (Molander and Grant, 1986) and suggests that in the dorsal horn, primary afferent input generates transsynaptic expression of c-fos-LI whose laminar distribution is related to the nature of the sensory stimulus.

In agreement, a pattern of Fos-LI consistent with the known nociceptive primary afferent input from the hindpaw was observed following subcutaneous administration of formalin in both awake, freely moving and anaesthetised rats (Presley *et al,* 1990; Leah *et al,* 1992). In the ipsilateral L4-5 dorsal horn, dense labelling was recorded in the superficial layers (laminae I and II) and in deeper layers (lam IV and V); (areas that contain large populations of nociceptive neurons), whereas moderate labelling was found in deeper laminae. The pattern of increased Fos-LI was time-dependent, reaching a maximum level at 2-3 hours, decaying to basal levels within 6 hours after the stimulus. However, upon sensitisation of the dorsal horn neurons, induced by prior peripheral noxious stimuli administered to the contralateral paw, the number of Fos-LI nuclei produced in both the ipsilateral and contralateral cord was significantly greater and outlasted the stimulus for several hours (Leah *et al,* 1992). This suggests that Fos may switch on genes coding for proteins that increase the sensitivity of the spinal neurons to further afferent input.

Indeed, the participation of c-fos in the peripheral sensitisation associated with intraplantar injection of carrageenan has been documented. By Northern Blotting, Draisci and Iadarola, (1989) demonstrated that a rapid increase in c-fos mRNA occurred in the lumbar spinal cord within 30 minutes of carrageenan injection. The content of c-fos mRNA rose dramatically and remained at peak elevation for at least 2 hours after injection of the inflammatory agent, returning to control levels by 8 hours. By using ISHH and immunocytochemistry methods to

label Fos and Fos-related proteins, 3 days after carrageenan injection, the induced Fos-IR was found in the nuclei of spinal cord neurons located predominantly in laminae I-II and V-VI (Noguchi *et al,* 1991,1992).

The expression of Fos has also been demonstrated in rat spinal dorsal horn after induction of longer-term hyperalgesic states, such as adjuvant induced arthritis (Menetrey *et al,* 1989; Hylden *et al,* 1992; Abbadie and Besson, 1993; Tolle *et al,* 1994) and the development of a neuroma following nerve injury (Chi *et al,* 1993).

In an acute study of adjuvant-induced arthritis in the rat, 16 hours after unilateral injection of CFA, the subcutaneous inflammation was accompanied by an increased concentration of c-fos-IR neurons in laminae I and II, around the base of the dorsal horn and in deeper laminae VII, VIII and X (Menetrey *et al,* 1989). After capsaicin treatment, this inflammation-induced increase was shown to be greatly attenuated (Hylden *et al,* 1992) indicating the input from small diameter afferents is important for the stimulus- induced increase in Fos-LI. More recently, the c-fos expression has been studied in a model of chronic CFA-induced polyarthritic rats for up to 22 weeks (Abbadie and Besson, 1993). C-fos expression followed the time course of arthritis and peaked simultaneously with hyperalgesia 3 weeks after innoculation, However, these results are in disagreement with those of Tolle *et al,* (1994), who showed in a monoarthritic model, that c-fos-IR neurons were completely absent after 2 weeks when hyperalgesia was still demonstrable, suggesting that c-fos is vital in the initial stages but not in the maintenance of the inflammatory state. However, there is a strong correlation between pain behaviour and the number of cells expressing Fos (Presley *et al,* 1990). Moreover, morphine pre-treatment produces a dose-dependent suppression of Fos expression which corresponds with its analgesic effects (Presley *et al,* 1990; Tolle *et al,* 1991). Although these observations do not necessarily mean that c-fos is involved, there is growing evidence for an association in central sensitisation and persistent nociception, the noxious stimuli producing Fos expression also induces behavioural hyperalgesia (Coderre and Melzack, 1987; Coderre *et al,* 1990), coinciding with the time course of Fos expression induced by carrageenan (Draisci and Iadarola, 1989). The concurrence of Fos expression and behavioural hyperalgesia is also evident in cases where a peripheral stimulus initiates but does not apparently maintain the hyperalgesia, e.g. heat injury of a rat's hindpaw (Coderre and Melzack, 1987) which produces an initial hyperalgesia in the ipsilateral hindpaw after injury in addition to a delayed hyperalgesia (after 4-24 hrs) in the contralateral hindpaw, coinciding with two

'waves' of Fos expression (Williams *et al,* 1990), also suggestive of a role of Fos expression in neural plasticity. This is supported by the observation that both the contralateral hyperalgesia (Coderre and Melzack, 1987) and the Fos expression in the contralateral dorsal horn (Williams *et al,* 1990) still develop when the injured limb was locally anaesthetised shortly after the injury.

After peripheral nerve injury induced by sciatic nerve transection, a persistent elevation in Fos-LI was induced predominantly in laminae I-II and V-VII of the ipsilateral lumbar enlargement of the spinal cord (Chi *et al,* 1993), demonstrating that peripheral nerve injury, induces a prolonged increase in Fos expression in neurons predominantly in those regions of the spinal cord that are associated with the transmission of nociceptive messages and thus this pattern of Fos-LI is probably the result of persistent neuronal activity in the spinal cord.

1.9.2 Consequences of c-fos Expression.

The Fos protein forms a heterodimer with Jun which binds to AP-1 like elements to form a DNA binding site in the promotor region of its target gene (Morgan and Curran, 1989). Several late onset (peptide precursor) genes have been reported to be activated by nociceptive inputs to dorsal horn neurons and it seems likely that the early induction of c-fos expression in these cells may fulfil some role in mediating these responses. Peripheral inflammation, noxious thermal stimulation, trigeminal nerve stimulation or nerve lesions result in the increase in the expression of mRNA encoding dynorphin (Draisci and Iadarola, 1989; Ruda *et al,* 1989; Naranjo *et al,* 1991; Hylden *et al,* 1992), enkephalin (Iadarola *et al,* 1988; Noguchi *et al,* 1992), SP (Noguchi *et al,* 1988; Minami *et al,* 1989; Pretel and Piekut, 1991) and glutamate (Kehl *et al,* 1991) in the dorsal horn or dorsal root ganglion.

The preprodynorphin gene encodes a family of structurally-related opioid peptides that have a high affinity for the κ opiate receptor (Corbett *et al,* 1982). Dynorphin-expressing neurons are thought to be involved in mechanisms that modulate pain sensations (Iadarola *et al,* 1988) in rostrally projecting neurons that transmit nociceptive signals e.g. SMT and perhaps STT. Furthermore, there is strong evidence suggesting that the preprodynorphin gene is a target for c-fos, co-induction of c-fos and preprodynorphin genes is reported to occur in the same neuronal elements predominantly in laminae I-II and V-VI of the spinal cord after noxious thermal stimulation (Naranjo *et al,* 1991; Draisci and Iadarola, 19898) and inflammation (Draisci and Iadarola, 1989; Hylden *et al,* 1992). Iadarola and colleagues (1988) have also found that the increase in Fos protein which peaks 2

hours after peripheral inflammation is followed by a large increase in preprodynorphin mRNA peaking at 3 days, followed by a subsequent increase in dynorphin peptide. Over 80% of the neurons in the superficial laminae and deeper dorsal horn which express preprodynorphin co-localise Fos-IR, which has been taken as evidence that Fos mediated signalling is coupled to dynorphin gene transcription (Dubner and Ruda, 1992). It has also been shown that phorbol esters which activate PKC, lead to a rapid induction of c-fos mRNA (within 30 minutes) and a later increase in the level of preprodynorphin mRNA (between 1.5 and 6 hours) in neuronal cells in culture (Naranjo *et al,* 1991). Also an AP-1 site has been identified in the preprodynorphin (Naranjo *et al,* 1991) promotor that binds fos/jun proteins which may therefore function as third messengers in the signal transduction mechanisms of pain processes.

Another possible target of c-fos is the preproenkephalin gene, but evidence is much more controversial than for dynorphin. While White and Gall, (1987) found a positive relationship between c-fos mRNA and the expression of the preproenkephalin gene in the rat hippocampus following seizure activity, others (Iadarola *et al,* 1988; Draisci and Iadarola, 1989) showed only a very small increase in preproenkephalin mRNA with c-fos protein in the spinal dorsal horn after peripheral inflammation, which did not produce measurable increases in enkephalin peptide content (Iadarola *et al,* 1988). The difficulty in measuring changes in preproenkephalin may be because there are high basal levels in the normal state and therefore it is difficult to detect any increase. However, more recently, it was found that peripheral inflammation induced an increase in preproenkephalin mRNA levels in spinal cord neurons and most neurons also localised Fos and Fos-related immunoreactivity (Noguchi *et al,* 1992). Furthermore, it has also been shown that Fos and Jun proteins bind to form an AP-1 like site in the promotor regions of the rat preproenkephalin gene (Sonnenberg *et al,* 1989).

Therefore it is possible that dynorphin and perhaps enkephalin are involved to some extent in noxious stimulus induced plasticity. As enkephalin typically produces inhibitory effects (Vaught *et al,* 1982) it may provide a mechanism by which central plasticity and hyperalgesia are minimised (Dubner, 1991; Sullivan and Dickenson, 1991). Dynorphin which is typically found to produce moderate antinociceptive effects (Hayes *et al,* 1987) has complex, dual effects in the spinal cord (Hylden *et al,* 1991) leading to the suggestion that it may produce direct excitatory effects on spinal projection neurons whilst producing

inhibition by a negative feedback mechanism on dynorphin containing neurons (Dubner and Ruda, 1992).

Substance P is one of several transmitters which might provide input to modulate and regulate the expression of c-fos. SP is known to be present in small diameter primary afferents (Hokfelt *et al,* 1975b) and after cutaneous mustard oil application, which stimulates small diameter afferents, c-fos expression was evoked in laminae I and II of the lumbar spinal cord (Pretel and Piekut, 1991). Double label immunocytochemistry revealed that the majority of these c-fos like immunoreactive neurons received input from SP immunoreactive neurons (and also 5-HT and enkephalin neurons) (Pretel and Piekut, 1991).

Another potential candidate for the regulation of c-fos is the neuropeptide NKA. As SP and NKA are derived from the same gene (DeBiasi and Rustioni, 1988), are thought to be co-localised, are distributed in a similar manner throughout the spinal cord (Ogawa *et al,* 1985) and NKA is also believed to be involved in nociception (see section 1.7), it is feasible that NKA on its own or in conjunction with glutamate or other factors, may regulate the expression of c-fos in the spinal cord.

The involvement of glutamate in the modulation and regulation of c-fos is not so clear. Sharp *et al,* (1990) showed that the NMDA receptor agonist quinolinic acid induced c-fos mRNA and Fos-related antigens throughout the ipsilateral adult brain cortex in similar patterns and suggested that NMDA receptors mediate induction of Fos following cortical injury. This was supported by the demonstration that pre-treatment with the NMDA antagonist MK-801 significantly suppressed formalin-induced Fos-like immunoreactivity in the superficial dorsal horn (Kehl *et al,* 1991). However, others have found that the NMDA antagonists ketamine and MK-801 did not affect the distribution of Fos labelled neurons in the spinal cord following noxious heat stimulation (Tolle *et al,* 1991), indicating that in these circumstances, at least, other factors play a greater role.

1.10 AIMS OF CURRENT WORK

This study aims to investigate the potential role of tachykinins that activate NK_1 and NK_2 receptors in models of both acute and sustained excitation of spinal dorsal horn neurons. In addition, the changes in intracellular signalling and gene expression by which tachykinins might bring about sensitisation of dorsal horn neurons are also investigated. Various experimental models have been employed:-

(1) Electrophysiological Techniques

(a) Extracellular recordings were made from rat laminae III-V multireceptive dorsal horn neurons. Ionophoresis of selective NK_1 and NK_2 agonists and antagonists was used to investigate the action of tachykinins on responses to brief noxious and innocuous stimuli.

(b) An investigation was carried out on the contribution of NK_1 and NK_2 receptors in dorsal horn to the sustained neuronal activity in C afferents induced by the peripheral application of the chemical algogen mustard oil (reported to be a selective activator of C afferents).

(c) Using extracellular recordings from laminae III-V multireceptive dorsal horn neurons, the effects of NK_1 and NK_2 antagonists on sensory responses (both noxious and innocuous) were examined before and after cutaneous application of mustard oil, which induces central sensitisation and hyperalgesia.

(2) Biochemical Techniques

The intracellular mechanisms involved in the sensitisation of dorsal horn neurons are unknown. Experiments were conducted to address any role of protein kinase C (PKC) in sustained nociceptive responses of dorsal horn neurons using the following techniques:-

(a) Extracellular recordings were made from multireceptive laminae IV/V dorsal horn neurons. PKC inhibitors were ionophoretically administered whilst recording activity evoked by repeated cutaneous application of mustard oil.

(b) The subcellular translocation of PKC evoked in the spinal cord by cutaneous application of mustard oil was examined. In addition, the involvement of NK_2 receptors was assessed using systemic administration of selective antagonists.

(3) *In Situ* Hybridisation Histochemistry

By use of *in situ* hybridisation histochemsitry, the induction of mRNA for the immediate early gene c-fos was used as a molecular marker to assess genomic responses of dorsal horn neurons. Repetitive cutaneous application of the C-fibre selective chemical algogen, mustard oil, was used to elicit sustained neuronal activity of dorsal horn neurons and the ensuing changes in induction of c-fos mRNA noted. The contribution of NK_1 and NK_2 receptors to this model of short-term sensitisation was assessed by intravenous administration of selective non-peptide antagonists.

(4) Behavioural Studies

A short study was undertaken to assess the role of NK_1 and NK_2 receptors in acute behavioural nociceptive models of tail-flick and paw-flick. These tests were repeated following a peripheral inflammatory stimulus (intraplantar carrageenan injection).

CHAPTER 2:
Electrophysiological Examination of Sensory Responses in Laminae III-V Rat Dorsal Horn Neurons.

2.1 AIMS

The tachykinins, substance P and neurokinin A are both present in fine somatosensory afferents and for some time substance P has been a candidate transmitter of nociception. However, there is increasing evidence that neurokinin A (and NK_2 receptors) play a crucial role in mediating spinal nociceptive transmission. Using the technique of local ionophoretic application of selective tachykinin receptor agonists and antagonists, whilst extracellularly recording from single superficial dorsal horn neurons, the possible role of NK_1 and NK_2 receptors in mediating brief cutaneous sensory responses was investigated.

2.2 MATERIALS

Animals: Male Wistar rats were obtained from Charles River UK Ltd, Margate, Kent, UK.

Anaesthetics: Alpha-chloralose and urethane were obtained from Sigma Chemical Company, Poole, Dorset, UK; Halothane (flurothane) was obtained from ICI Pharmaceuticals, Macclesfield, Cheshire, UK.

Laboratory Chemicals: Standard laboratory chemicals were of Analar grade and from the following suppliers: Sodium chloride (NaCl), DLH (D, L, homocysteic acid) and Pontamine Sky Blue (PSB) were obtained from Sigma Chemical Company, Poole, Dorset, UK; Agar was obtained from Oxoid Ltd, Basingstoke, Hampshire, UK.

Drugs: The tachykinin agonists, neurokinin A (NKA) & [Met-O Me^{11}] SP (SPOMe) were obtained from Cambridge Research Biochemicals, [N-acetyl-Arg^6,Sar^9,$Met(O_2)$]SP_{6-11} (Sar-9) was a gift from Professor D Regoli and [Lys^3,Gly^8-R-γ-lactam-Leu^9]neurokinin-(3-10), (GR 64349) from Glaxo Group Research.

The tachykinin antagonists, acetyl-Leu,Met,Gln,Trp,Phe-NH_2 (L-659,874); cyclo(Gln,D-Trp,Me-Phe,(R)Gly[ANC-2]Leu,Met)$_2$, (L-668,169) and cyclo(Gln,Trp,Phe,Gly,Leu,Met), (L-659,877) were obtained from Cambridge Research Biochemicals & [D-Pro^9[spiro-γ-lactam]-Leu^{10},Trp^{11}]physalaemin-(1-

11), (GR 82334) was a gift from Glaxo Group Research; Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2, (R396) was a gift from Professor D Regoli.

2.3 METHODS

2.3.1 Animals

Adult male Wistar rats (250-300g) were housed in standard rat cages in groups of 3 or 4, maintained under controlled lighting conditions (12 hour light : 12 hour dark); temperature (22-23°C) and allowed free access to food (rat and mouse standard maintenance diet) and tap water.

2.3.2 Surgery

The animals were briefly anaesthetised with halothane and following cannulation of the jugular vein, this was changed to an α-chloralose and urethane mixture (α-chloralose 60mg/kg and 1.2g/kg urethane: intravenous). The anaesthetic level was continuously monitored and supplemental doses given in 0.2ml aliquots as required on testing blink reflex and paw withdrawal. A tracheal cannula was inserted and secured by ties and the spinal cord was exposed by removal of surrounding muscle.

The animal was placed in a stereotaxic frame and secured in place by means of a mouth piece, earbars, a tail-bar and 3 pairs of swan-neck clamps placed carefully under the lateral processes of alternate vertebrae to support the vertebral column (see Figure 2.1). A laminectomy was performed under x20 magnification to expose T12-L4 for recording, the surrounding skin flaps were then tied to the frame in order to create a 'pool' (see Figures 2.1 and 2.2). A 2% agar solution at 40°C was then injected under the spinal bone at the rostral end of the laminectomy and poured over into the 'pool'. Once set, a section of agar was removed from over the spinal cord and the dura carefully removed. Paraffin at 37°C was then poured over the exposed cord to protect the surface once the dura had been extracted, (see Figure 2.2).

When surgery was completed the anaesthetic was changed to α-chloralose (60mg/kg) and 0.1ml aliquots were given as required. Rectal temperature and occasionally arterial blood pressure were continuously monitored throughout the experiments and maintained within physiological limits i.e. 37°C and 100-150mmHg respectively. Rats were allowed to respire spontaneously but

Figure 2.1

An outline of the methods used for extracellular recording from the dorsal horn of the spinal cord from anaesthetised rats.

A thoraco-lumbar laminectomy was performed under x20 magnification to expose T12-L4 for recording, 3 pairs of swan neck clamps supported the vertebral column. The surrounding skin flaps were then tied to the frame in order to create a 'pool'. A 2% agar solution was then injected under the bone at the rostral end of the laminectomy, which created a stable preparation (see Figure 2.2 for details). Extracellular recordings from single neurons were made via the central barrel of a 7-barrelled glass microelectrode filled with 4M NaCl. Multireceptive neurons (which had responses to both noxious and innouous stimuli) were used. Their cutaneous excitatory receptive fields were located on the ipsilateral hindlimb, paws and toes. The innocuous cutaneous stimulus used was a rotating brush, whereas the noxious cutaneous stimulus was provided by either a calibrated pinch or thermocouple-controlled radiant heat-lamp (see Figure 2.3 for details of cutaneous stimulators used). Mustard oil (8% in paraffin oil) was applied to the receptive field area using a paintbrush and the sensory responses then repeated.

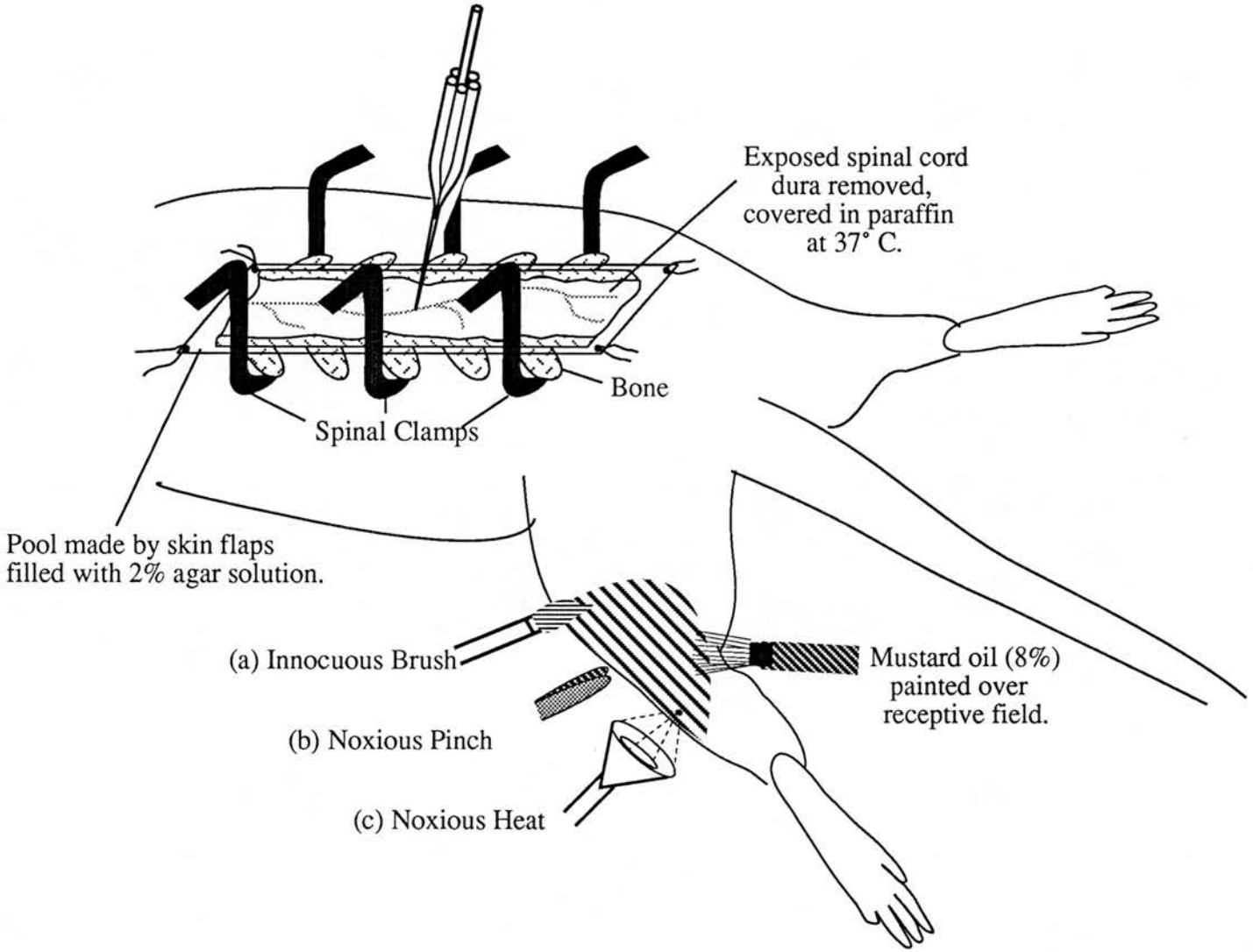

Exposed spinal cord
dura removed,
covered in paraffin
at 37° C.
Bone
Spinal Clamps
Pool made by skin flaps
filled with 2% agar solution.
(a) Innocuous Brush
(b) Noxious Pinch
(c) Noxious Heat
Mustard oil (8%)
painted over
receptive field.

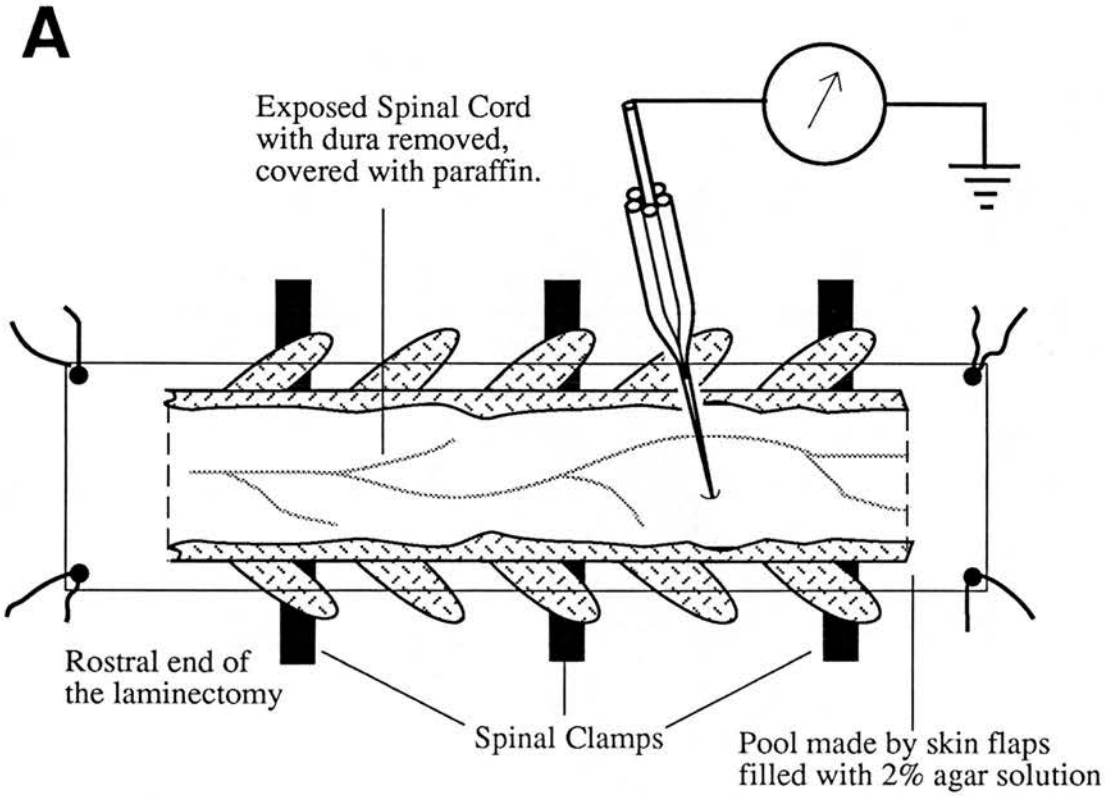
Figure 2.2

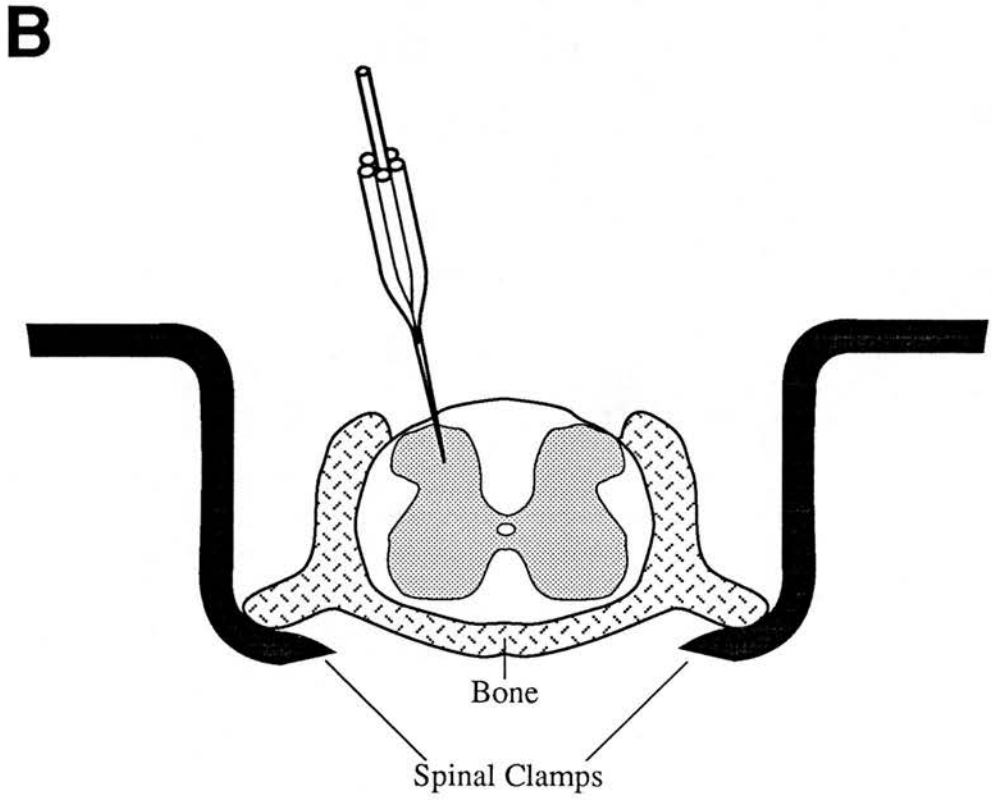
The thoraco-lumbar laminectomy

A schematic diagram of (A) a dorsal view and (B) a transverse view of the rat thoraco-lumbar laminectomy and the preparative surgery used for extracellular neuronal recording.

A laminectomy was performed to expose T12-L4 for recording, 3 pairs of swan neck clamps supported the vertebral column. A 2% agar solution was carefully injected 0.5-1.0cm beyond the spinal cord and intact dura and further agar covered the exposed spinal cord and filled the pool surrounding the laminectomy. This procedure improved the stability of the spinal cord, facilitating maintained extracellular recordings from dorsal horn neurones. Once set, a section of agar was removed from over the spinal cord and the dura carefully removed. Paraffin oil at 37°C was then poured over the exposed cord to protect the surface.

Extracellular recordings were made from single neurons in the superficial dorsal horn found at depths below dorsal surface between 0-650μm (as stated on the microdrive) via the central barrel of a 7-barrelled glass microelectrode filled with 4M NaCl. The other 6 barrels were used for ionophoresis and contained various tachykinin agonists and antagonists.


A
Exposed Spinal Cord
with dura removed,
covered with paraffin.
Rostral end of
the laminectomy
Spinal Clamps
Pool made by skin flaps
filled with 2% agar solution


B
Bone
Spinal Clamps

humidified oxygen was supplied to the area of the tracheal cannula to enrich the inspired air.

2.3.3 Recording and Ionophoresis

Extracellular recordings from single neurons found at depths below dorsal surface between 0-650μm (as stated on the microdrive) were made via the central barrel of a 7-barrelled glass microelectrode filled with 4M NaCl, (pH 4.0-4.5), (see Figures 2.1 and 2.2). Electrode tip sizes were 4.0-4.5μm and DC resistances were 5-8MΩ. The band-width of the recording amplifier was 1Hz -7 kHz. The electrode was secured in place by a metal arch placed over the preparation and moved by means of a microdrive system in 2 or 4μm steps until a suitable recording was obtained.

Raw data recorded from this central barrel was displayed on oscilloscopes. The other 6 barrels were used for ionophoresis and contained :- 1M NaCl (pH 4.0-4.5) for automatic current balancing and current controls (Neurophore Ionophoresis System, Medical Systems Corporation); Pontamine Sky Blue (2% in 0.5M sodium acetate) for marking recording sites by ejection at 100μ A for 10-13 minutes. (The laminar locations of recorded neurons were assessed from Pontamine Sky Blue Spots on cryostat sections, see later); DLH (100mM, pH 8.0-8.5), an internal control of neuron stability and responsiveness and a selection of the following drugs:-

TACHYKININ AGONISTS

Neurokinin A, (NKA) (1mM, pH4.0-4.5); [Met-O-Me^{11}]SP (SPOMe); (0.1mM in 0.3% dimethylformamide (DMF), pH 4.0-4.5): [N-acetyl-Arg^{6},Sar^{9},Met(O_2)] SP_{6-11}(Sar-9) (0.1mM in 0.3% DMF, pH 4.0-4.5) GR 64349 (1mM in distilled H_2O, pH 4.0-4.5) .

TACHYKININ ANTAGONISTS

L-659,874; L-668,169 and L-659,877 (0.1mM in 0.3% DMF, pH 4.0-4.5):GR 82334 (1mM in distilled H_2O, pH 4.0-4.5): R396 (0.1mM in 0.3% DMF, pH 4.0-4.5).

Drugs were either freshly made or stored at -20°C over dessicant and solutions once thawed, were not re-frozen, or used again. All drugs were applied with cathodal currents. Retaining currents of -10nA were used to minimise drug leakage between tests.

2.3.4 Histological Determination of Laminar Location

At the end of each experiment, the cord was carefully removed using a scalpel, watchmaker forceps and iris scissors. It was placed in a plastic weighboat, orientation marked and frozen with an aerosol spot-freezing spray. It was then fixed overnight in 10% formal saline and oriented to approximately 90° to the horizontal plane in 0.25% agar solution in a freezing microtome. 52μm sections were cut and collected in 0.1M phosphate buffer (pH 7.4). Once the Pontamine Sky Blue spot had been visually located, the appropriate section (with the blue spot on it) was mounted in phosphate buffer on a slide and sealed under a coverslip with varnish. It was left overnight to dry and then photographed. The section was then removed from the slide and remounted on a subbed slide, dried overnight in formalin vapour to ensure adhesion and then stained with neutral red and mounted under Depex Mounting Medium.

2.3.5 Quantification of Sensory Responses

Multireceptive neurons were used in these studies i.e. those which displayed responses to both noxious and innocuous stimuli. Neurons were initially found by their responsiveness to widespread manual brushing of the ipsilateral hindlimb. Cutaneous receptive fields of the neurons were first localised by excitatory responses to manual brush, then more restricted pinch and noxious heat receptive fields were located within this innocuous field, (see Figures 2.1 and 2.3).

All the multireceptive neurons used had excitatory receptive fields on the ipsilateral hindlimb, paws and toes (for an example see Figure 2.3). The controlled noxious and innocuous stimuli were applied to adjacent sites within the ipsilateral cutaneous field. The innocuous cutaneous stimulus used was either a motorised, rotating brush or occasionally manual brushing using a small soft paintbrush (10-15 second period). The noxious cutaneous stimuli were provided by either a calibrated pinch (serrated forceps with a graduated controlled displacement) or a thermocouple-controlled radiant heat lamp, precisely raising skin surface temperature from 30°C to 46-48°C usually over 3 seconds rise time, for a controlled period of 5-10 seconds (see Figure 2.3).

The foot was fixed in position by attachment to a clamp such that the cutaneous stimulators remained in exactly the same position for the duration of the test. Each set of stimuli was cycled over a minimum 3 minute period to allow full recovery of the neuron between tests and to avoid unnecessary sensitisation of any

Figure 2.3

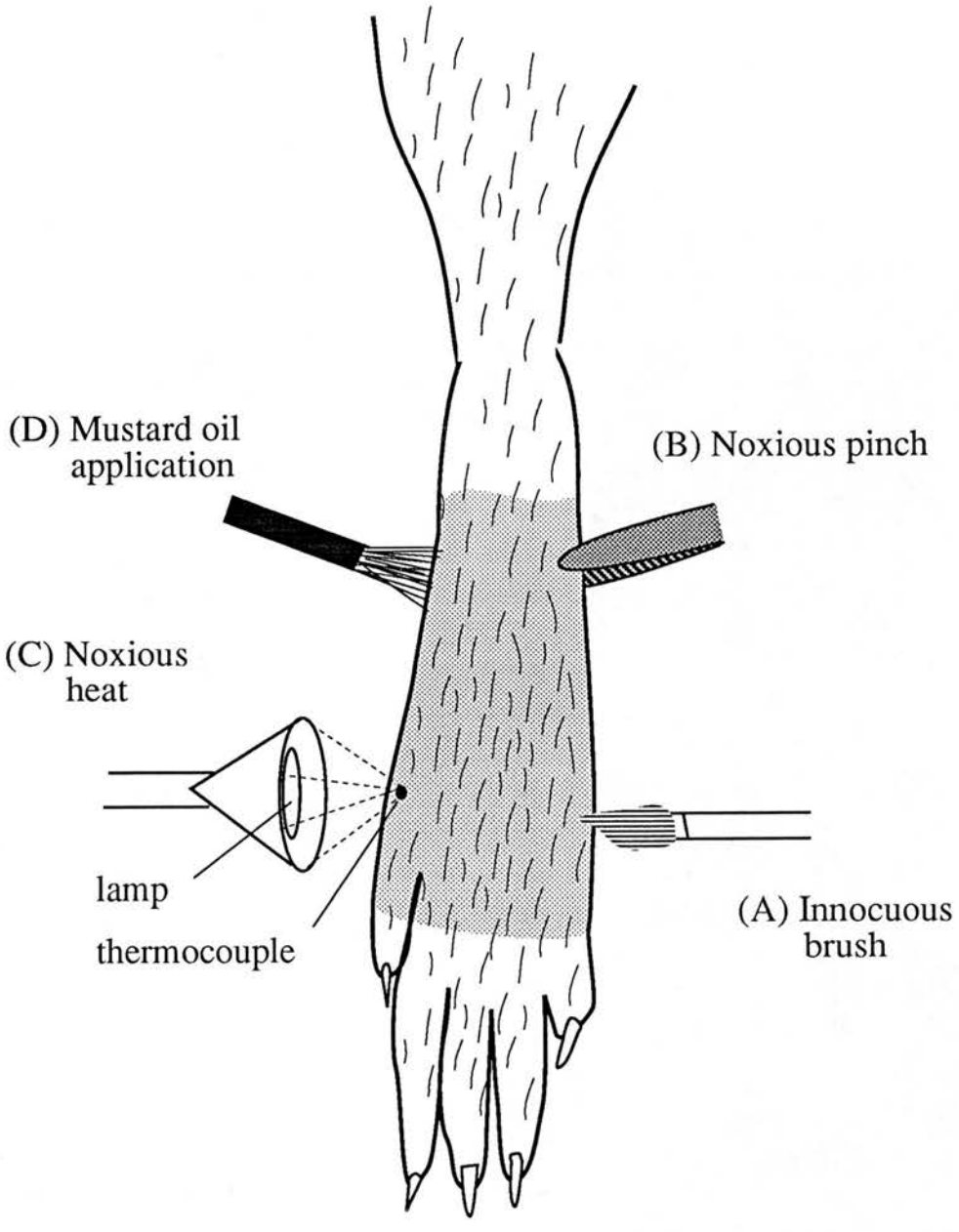
Diagram of cutaneous stimulators used and mustard oil application

(a) Innocuous Brush- This was provided by either a soft motorised, rotating brush or occasionally manual brushing using a small soft paintbrush for a 10-15 second period.

(b) Noxious Pinch- This was quantifiable by means of a graded scale which directly allowed the pincher to be closed to the same level on each test, for a duration of 10 seconds.

(c) Noxious Radiant Heat- This was provided by a thermistor-controlled radiant heat lamp. The surface temperature was measured by a thermocouple, in the centre of the heated area, placed within 1-2 cm of the cutaneous receptive field. The temperature was raised from 30°C to 46°C to 48°C within 5 seconds and held at 46 to 48°C for a duration of 5 to 10 seconds.

(d) Mustard Oil Application- The algogen (usually 8% in paraffin oil) was painted over the receptive field area with a soft paintbrush.


(D) Mustard oil application
(B) Noxious pinch
(C) Noxious heat
lamp
thermocouple
(A) Innocuous brush
Hairy surface of paw
Low threshold receptive field for mustard oil application.

of the responses (see Figure 2.4). Throughout the tests, care was taken to ensure that the recorded spike height remained significantly greater than the field potentials; occasionally this meant moving the position of the recording electrode by a few μm in order to get closer to the neuron during the testing procedure.

First, control responses to each of the cutaneous stimuli were assessed. This was repeated once or twice and only if the degree of variability was very small (10% maximum) were the tests continued. Each drug was ionophoresed for 1 minute before the start of each test cycle and continued throughout the cycle, starting at a low ejection current and increasing usually by 10nA, stepwise, each cycle. After each drug application, full recovery of responses was achieved (from 5-30 min) before the next drug was tested (see Figure 2.4 for a diagramatic representation).

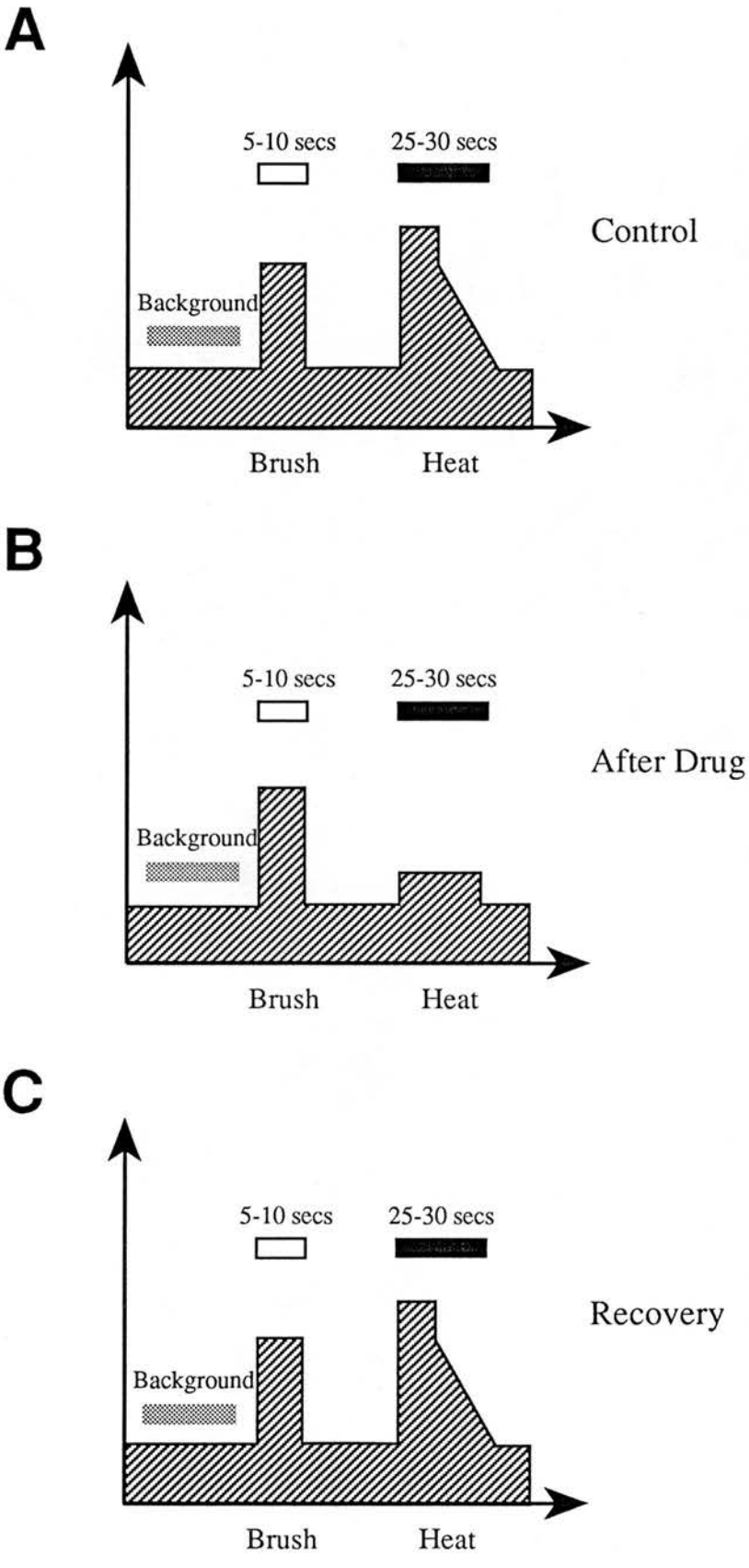
2.3.6 Analysis

The neuronal firing was continuously recorded on FM tape (Racal) and firing rates were plotted on-line by computer (IBM PS/2-70-121) together with stimulator and ionophoresis markers. The data was stored on the hard-disc of the computer and was analysed off-line by integrating the number of stimulus-induced action potentials in selected constant time intervals, taking spontaneous activity prior to the responses into account (by its subtraction from each sensory response recorded). Responses to mechanical brush and pinch were integrated over the 5-10 second stimulus times whereas responses to noxious thermal stimuli were integrated over 25-30 seconds from the start of the 48°C plateau, to include the long-latency activity often evoked by this stimulus (see Figure 2.4). These responses were expressed as percentages of control values. Ionophoresis of the direct excitant, D, L-homocysteic Acid (DLH) at the same currents used for ionophoresis of agonists/antagonists was used to ensure that any drug responses were not due to movement of the recording electrode away from or closer to the neuron under study. The response to ionophoresis (at the same current) of DLH remained constant throughout the experiment.

Figure 2.4

A typical example of the analysis technique used to study the ongoing firing frequency records achieved during electrophysiological recording from a lamina III-IV multireceptive neuron.

(a) shows the typical, well-matched control responses for the sensory stimuli under investigation. For each of brush and heat, the number of stimulus-induced action potentials in selected constant time intervals (10-15 seconds for brush and 25-30 seconds for heat) were integrated. This procedure was repeated after ionophoresis of the drug under study (b) and again after recovery from the drug (c).


A
5-10 secs
25-30 secs
Control
Background
Brush
Heat
B
5-10 secs
25-30 secs
After Drug
Background
Brush
Heat
C
5-10 secs
25-30 secs
Recovery
Background
Brush
Heat

2.4 RESULTS

2.4.1 Characteristics and Receptive Field Properties of Neurons

The present results were obtained from 61 neurons located in laminae III-V of the dorsal horn. Their position was generally at a depth of 300-800μm as read on the microdrive and their laminar position was confirmed by histological identification in transverse sections of the spinal cord. The positions of Pontamine Sky Blue (PSB) marks are shown in Figure 2.5. Occasionally the PSB histological identification could not be found, this is most probably due to poor extraction of the spinal cord, causing damage or alternatively the ionophoretic apparatus was defective.

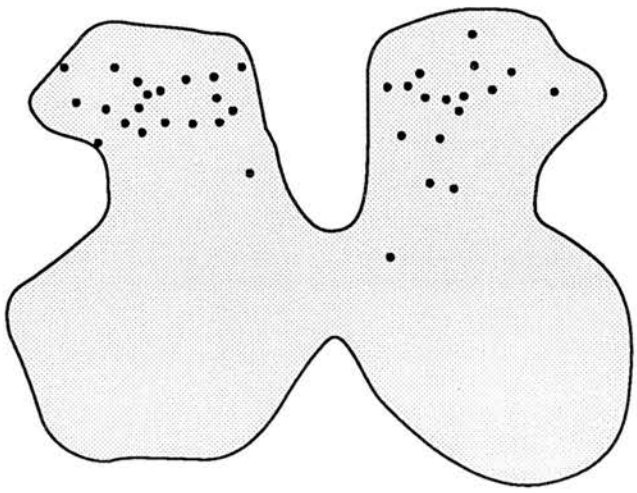
All neurons described were multireceptive i.e. responded to both noxious and non-noxious stimuli (noxious heat, noxious pinch and non-noxious brush). Care was taken not to overheat the noxious heat receptive field site as this could lead to sensitisation and damage. Neurons which had mismatched responses to stimuli (i.e. a very strong response to one stimulus and a very weak response to another) were discarded as these could not be relied upon to give accurate results. Also, neurons which did not give constant responses over the control testing were not used.

Generally, the spontaneous activity of the cells under examination was very low (less than or equal to 1 Hz) but on occasions it was a little higher (5-8 Hz). With these cells, care had to be taken that the sensory responses were clearly distinguishable from background activity. All neurons had cutaneous receptive fields located on either the paws (hairy or glabrous skin), toes or flank of the ipsilateral hindlimb (see Figures 2.1 and 2.3). These were mapped using innocuous stimuli and monitored continuously throughout the experiment to ensure no change in size ensued, generally they remained topographically constant throughout the recording period. The size of the receptive fields varied considerably between different neurons under examination, from the nail-bed of one toe to the whole flank of the ipsilateral limb, however, this did not seem to have any detectable effect on the recording characteristics of neurons (or the drug effect thereafter). Ionophoresis of the direct excitant, D, L-homocysteic Acid (DLH) at the same currents used for ionophoresis of agonists/antagonists was used to ensure that any drug responses were not due to movement of the recording electrode away from or closer to the neuron under study. The response to ionophoresis (at the same current) of DLH remained constant throughout the experiment.

Figure 2.5

Camera lucida plot of the recording sites of dorsal horn neurons studied

Extracellular recording sites were marked with Pontamine Sky Blue at the end of each experiment; a typical exmple is depicted by an asterix in the photograph. A transverse section was taken for histological examination and a camera lucida drawing was made of the location of some of these neurons, as represented in the diagram by black circles.



2.4.2 Current and Vehicle Controls

Neurons under consideration were routinely tested with vehicle and current controls. They were examined under identical conditions to the agonists and antagonists under study with ejection for at least 1 minute increasing the current in a 10-20 nA stepwise manner every consecutive minute.

The vehicle required for certain compounds (0.3% DMF in water), had no effect in 3 out of 3 neurons when ejected at up to 80nA for 12 minutes.

2.4.3 Control Responses to Sensory Stimuli

Before the drug effect on a neuron could be tested, a reproducible set of control responses to sensory stimuli had to be achieved. A minimum of two consistent cycles of sensory responses was always achieved before testing was started. It was always important to ensure that the sensory stimulators were in stable positions and were unlikely to move during the course of the experiment. A three minute cycle of responses was generally followed to minimise any sensitisation of responses to repetitive stimulation. On occasions this was increased to 4 or 5 minutes depending upon the neuron under study.

2.4.4 Effects of Ionophoretically Applied Neurokinin Antagonists

(a) Ionophoretically Applied NK_1 Antagonists

The effects of three ionophoretically-applied NK_1 antagonists ejected close to multireceptive dorsal horn neurons in laminae III-V of the spinal cord were assessed. The highly-selective NK_1 antagonists used were L-668,169, GR 82334 and [D-Pro^4,D-Trp^{7,9,10},Phe^{11}]substance P-(4-11), which were tested as previously described. Once the control responses had been tested, the drug under study was ionophoresed for 1 minute before the sensory responses were tested and then currents used to release the drug was increased in a stepwise manner.

In the majority of neurons tested* there were consistent results with no discernible effect on spontaneous activity but with a clear increase in innocuous brush and no significant change in responses to either noxious pinch or heat.* (12/14)

Ionophoresis of L-668,169

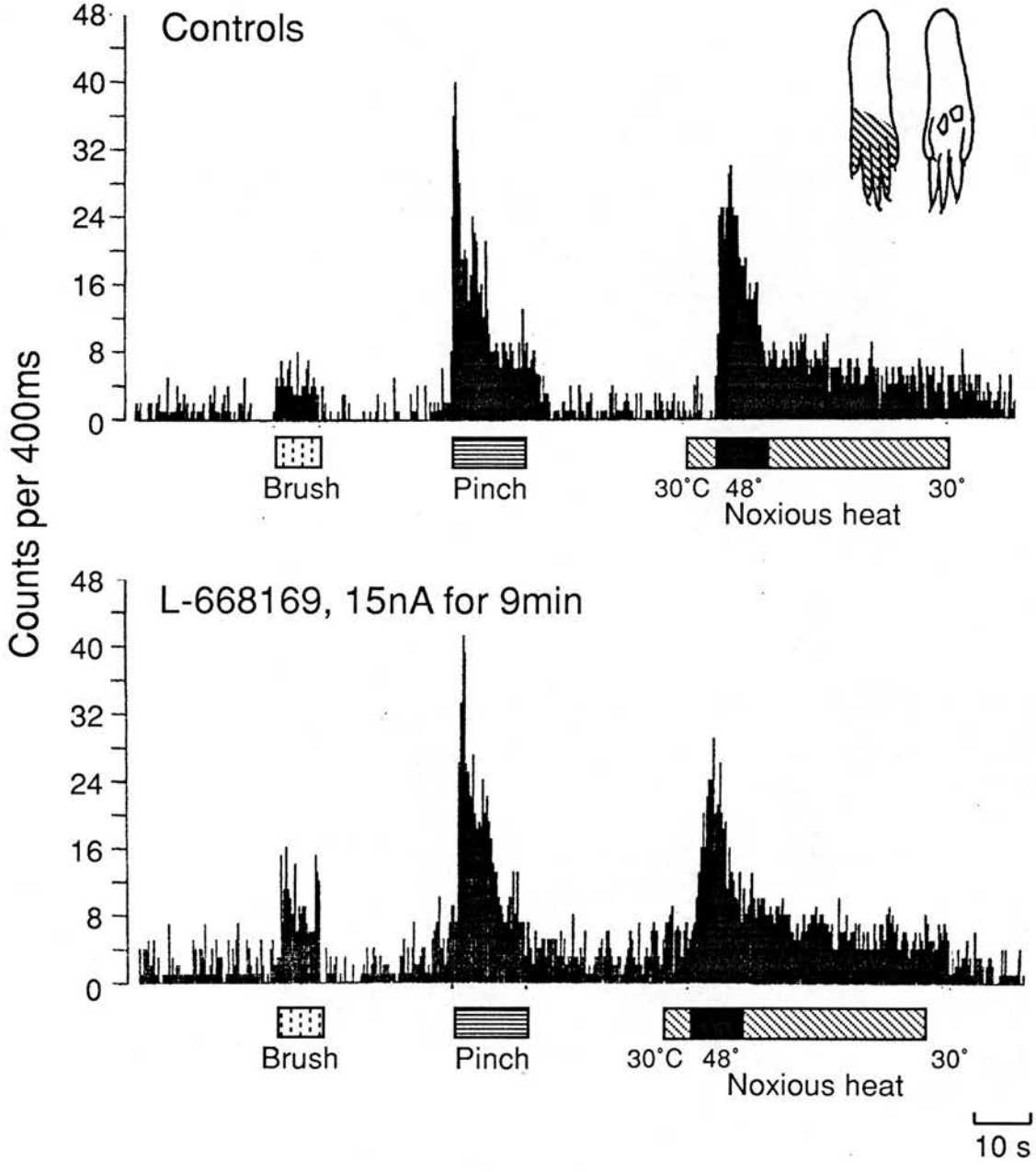
Eight multireceptive dorsal horn neurons in laminae III-V were tested with L-668,169 (Regoli *et al,* 1987). Figure 2.6 shows the ongoing firing records showing typical effects of ionophoretically-applied L-668,169 on somatosensory responses of these neurons. In 6/8 neurons tested, ionophoresis of the NK_1

Figure 2.6

Ongoing firing frequency records showing typical effects of the NK_1 receptor antagonist L-668,169 on somatosensory responses of laminae III-V neuron.

A modest facilitation of responses to innocuous brush was observed in 6 out of 8 neurons tested, with no other consistent changes. Recovery from drug effects was not examined closely, but in 3 out of 4 cells examined, responses to brush had recovered to within ± 15% of control levels within 9-24 min after stopping ionophoresis of the drug. The fourth cell showed approximately 50% recovery within 4 min. The inset shows the position of the cutaneous receptive field on the ipsilateral hind-limb. The duration of each of the stimuli is indicated, in the case of noxious heat, this representing the period for which the skin temperature exceeded the generally accepted noxious threshold of 45°C.

This diagram represents ongoing firing frequency records from a single laminae III-V dorsal horn neuron, a five minute recovery period was attained before ionophoresis of L669-169 was initiated. Full recovery was observed 12 minutes after stopping ionophoresis of the drug.


Controls
48
40
32
24
16
8
0
Brush
Pinch
30°C 48°
30°
Noxious heat
L-668169, 15nA for 9min
Counts per 400ms
10 s

receptor antagonist at 20nA for 6 minutes resulted in a small but consistent enhancement of activity evoked by innocuous brush to 141 ± 9% of mean pre-drug control (mean ± s.e.m., $P<0.05$, Wilcoxon Test). There was little effect on background (spontaneous) activity (which was 95 ± 7% of pre-drug activity) or responses to noxious pinch or heat (which had stimulus-evoked neuronal firing rates of 105 ± 10 and 82 ± 9% of mean pre-drug controls respectively). In the remaining two cells, ionophoresis of L-668,169 at 20nA for 6 minutes resulted in no detectable changes. In 4 cells where recovery was assessed there was 50% (n=1) and over 85% (n=3) reversal of the effect of L-668,169 on brush responses by 4 and 9-24 minutes respectively after cessation of ionophoresis. The vehicle (0.3% DMF in water) had no effect in 3 out of 3 neurons when ejected at 80nA for 12 minutes. Records such as those shown in Figure 2.6 were analysed off-line by integrating the number of stimulus-induced action potentials in selected constant time intervals, taking spontaneous activity into account. The values were calculated as a percentage of the mean pre-drug control value and are expressed as the mean control value. Statistical significance of the changes was assessed by the Wilcoxon test and the data is summarised in Figure 2.7.

Ionophoresis of GR 82334

The ionophoretic effects of the NK_1 receptor antagonist GR 82334 (Hagan *et al,* 1991) were investigated in 6 multireceptive neurons in laminae III-V of the spinal dorsal horn. In 5/6 cells tested with a mean ejection current of 32 ± 4nA over 6 minutes there was a small but significant increase in innocuous brush to 131 ± 8% of pre-drug control levels ($P<0.05$, as assessed by the Wilcoxon Test) with no significant effect on spontaneous activity (103 ± 8% of pre-drug spontaneous activity) or noxious heat, (after 1 minute of GR 82334 ejection the response to noxious heat was 91 ± 11% of pre-drug control value). Noxious pinch was not examined in this set of experiments, (see Figure 2.8 for a summary of the results). In the four cells tested, recovery of the response to innocuous brush was observed 3 minutes after termination of ionophoresis in all cells. Figure 2.9 shows ongoing firing frequency records demonstrating typical effects of the NK_1 receptor antagonist GR 82334 on somatosensory responses of a laminae III-V neuron, with recovery observed after 3 minutes.

Figure 2.7

Summary of the effects of neurokinin receptor antagonists and agonists on laminae III-V neurons.

All values are calculated as a percentage of the mean pre-drug control value and are expressed as the mean ± s.e.m. Responses were designated as being essentially unchanged if they remained within 20% of the mean control value. Neurons in the population tested which were not influenced in the characteristic predominant fashion illustrated, all showed no (or no consistent) changes in their responses. For the sub populations of neurons affected by the drugs, the statistical significance of changes was assessed by Wilcoxon test (a = $p<0.05$).

Drug and Conditions		Stimulus-evoked neuronal firing rate (% of mean pre-drug control)					
		Spontaneous	Brush	Pinch	Noxious heat	Number of neurons influenced.	Number of neurons tested.
Antagonists							
L 668,169 (NK_1)	20 nA for 6 min.	95 ± 7	141 ± 9[a]	105 ± 10	82 ± 9	6	8
GR 82334 (NK_1)	32 ± 4nA for 6 min.	103 ± 8	131 ± 8[a]	-	91 ± 11	5	6
L 659,874 (NK_2)	10 nA for 6 min.	93 ± 7	103 ± 9	99 ± 8	51 ± 6[a]	8	10
	20nA for 6 min.	95 ± 12	104 ± 6	85 ± 11	34 ± 5[a]	9	9
Agonists							
[Ac-Arg^6,Sar^9, $Met(O_2)^{11}$] -substance P-(6-11) (NK_1)	31 ± 4 nA for 5 ± 1 min	121 ± 13	52 ± 5[a]	98 ± 5	92 ± 6	9	13
	57 ± 5 nA for 6 ± 2 min	536 ± 88[a]	67 ± 9[a]	91 ± 14	106 ± 11	6	6
Neurokinin A (NK_2)	11 ± 5 nA for 7 ± 1 min	112 ± 10	89 ± 11	108 ± 10	155 ± 13[a]	6	6
	44 ± 4 nA for 6 ± 1 min	354 ± 61[a]	79 ± 19	120 ± 21	64 ± 16[a]	6	8
GR 64349 (NK_2)	66 ± 6 nA for 7 ± 1 min	398 ± 54[a]	111 ± 13	88 ± 20	75 ± 11[a]	5	5

Figure 2.8

Time dependence of the effects of neurokinin receptor antagonists on thermal nociceptive responses.

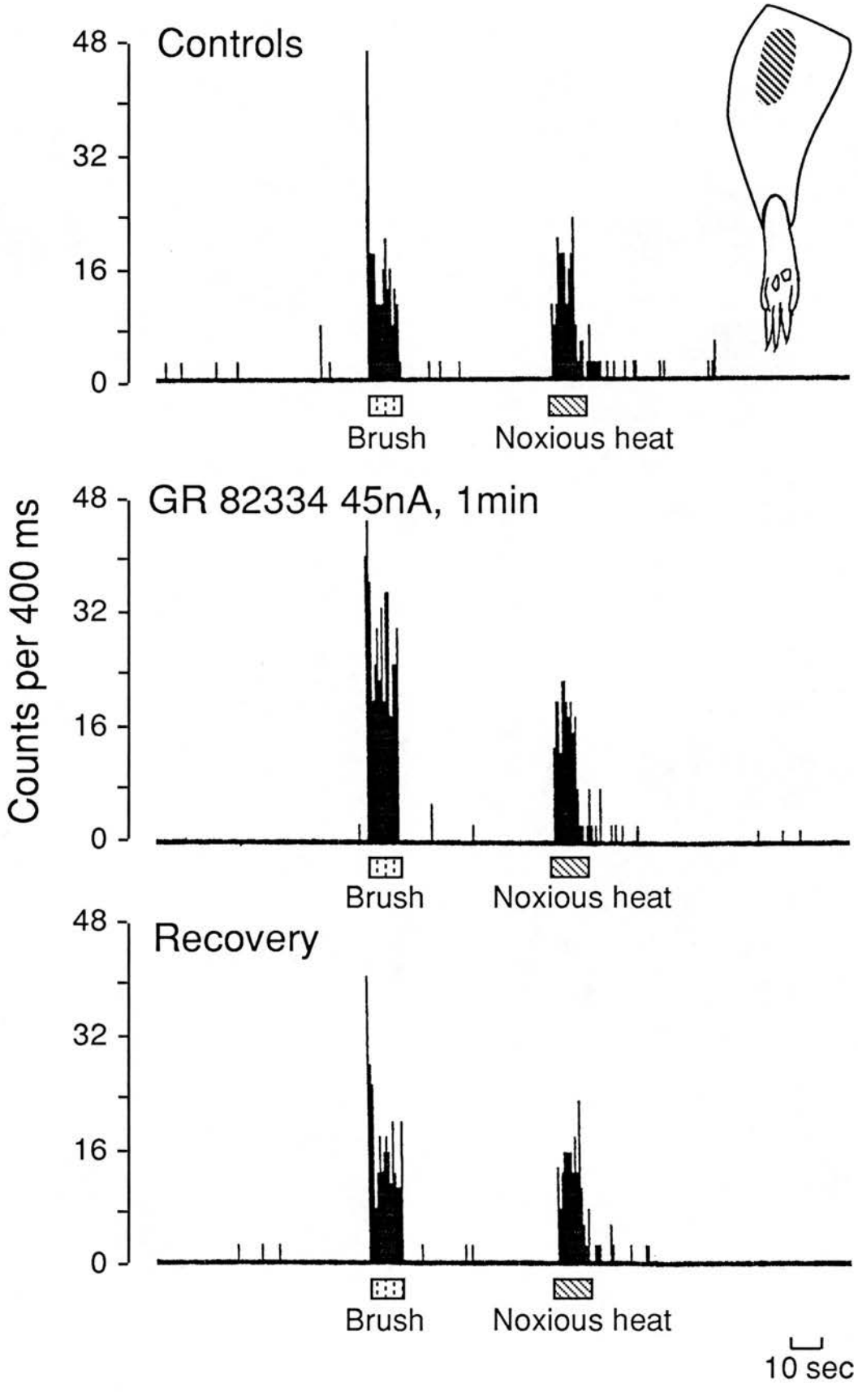
All values represent evoked activity (corrected for background) and are shown as percentages of the mean stimulus-induced activity occurring during the stimulus. Values are expressed as mean ± s.e.m.

	Noxious heat-induced increase in neuronal firing rate (%of control response during stimulus)		
	During Stimulus	0-10s after stimulus	10-20s after stimulus
(a) L668,169 (n=6)			
Control	100 (11± 1s)	21 ± 7	15 ± 9
Drug (20nA for 6 min)	90 ± 6	20 ± 11	9 ± 7
(b) GR 82334 (n=5)			
Control	100 (10s)	28 ± 7	9 ± 5
Drug (32nA for 6 min)	93 ± 10	23 ± 12	8 ± 7
(c) L 659,874 (n=9)			
Control	100 (11 ± 1s)	25 ± 9	11 ± 9
Drug (20nA for 6 min)	36 ± 6	10 ± 6	7 ± 7

Figure 2.9

Ongoing firing frequency records showing typical effects of the NK_1 receptor antagonist GR 82334 on somatosensory responses of a laminae III-V neuron.

Facilitation of responses to innocuous brush (with no other consistent change) was observed in 5 out of 6 cells examined. The lower trace shows recovery 3 min after termination of ionophoresis. Similar effects were observed in 3 further cells where recovery was assessed. The inset shows the position of the cutaneous field on the ipsilateral limb.


Controls
48
32
16
0
Brush
Noxious heat
GR 82334 45nA, 1min
Counts per 400 ms
Recovery
10 sec

Ionophoresis of [D-Pro^4,D-Trp^{7,9,10},Phe^{11}] substance P-(4-11)

The action of NK_1 receptor antagonists on multireceptive laminae III-V dorsal horn neurons was confirmed with a brief study using a further selective NK_1 antagonist [D-Pro^4,D-Trp^{7,9,10},Phe^{11}] substance P(4-11) (Regoli *et al,* 1987). Only 2 neurons were investigated, however the action of this NK_1 antagonist mirrored that of the two other NK_1 antagonists studied (L-668,169 and GR 82334).

In 2 out of 2 neurons tested with [D-Pro^4,D-Trp^{7,9,10},Phe^{11}] substance P-(4-11) (30-40nA, 6 min), the somatosensory responses to brush were increased by 33 and 41% of pre-drug activity with no consistent change in spontaneous activity, noxious heat or pinch.

Ionophoretic currents of up to 50, 45 and 80nA were tested for L-668,169, GR 82334 and [D-Pro^4,D-Trp^{7,9,10},Phe^{11}]substance P-(4-11) respectively, until either spike reduction or technical factors prevented further study. The effects on sensory responses at these currents were similar to those at lower currents, notably there still being no consistent inhibition of responses to noxious heat.

(b) Ionophoretically-Applied NK_2 Antagonists

The peptide antagonist L-659,874 (which is highly selective for NK_2 receptors (McKnight *et al,* 1988)) was tested by ionophoresis close to the recorded cells. The rather more potent cyclic analogue of L-659,874, L-659,877 could not be tested because of its low solubility in acceptable vehicles. Effects of L-659,874 were tested on multireceptive neurons as previously described in the methods section. Once the control responses had been obtained for both noxious and innocuous cutaneous stimuli, the NK_2 antagonist was ionophoresed for 1 minute before and during the cutaneous somatosensory tests which followed. Recovery was observed before further testing was continued.

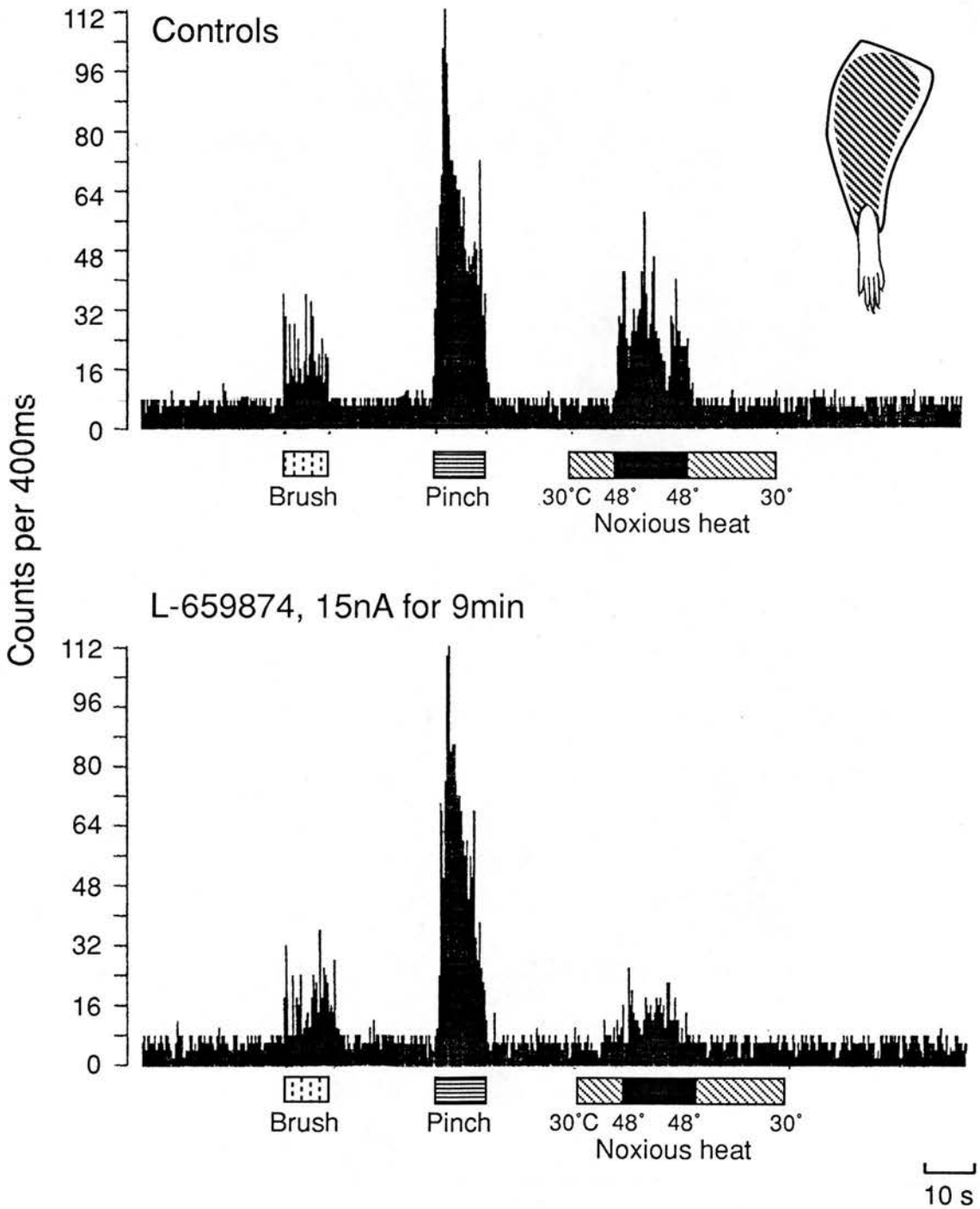
Ionophoresis of L-659,874

L-659,874 produced a very different response from the NK_1 antagonists tested. A typical response to ionophoresis of L-659,874 is depicted in Figure 2.10; a highly selective inhibition of responses to noxious thermal stimuli without altering responses to noxious pinch, innocuous brush or spontaneous activity. This was seen in 8/10 neurons tested by ejection of 10nA of L-659,874 for 6 minutes. By analysing records off-line and assessing the values as a percentage of

Figure 2.10

Ongoing firing frequency records showing typical effects of the NK_2 receptor antagonist L-659,874 on somatosensory responses of a laminae III-V neuron.

A marked attenuation of responses to noxious heat but not other stimuli or spontaneous activity was seen in all 11 neurons tested at currents of 15-20nA. Recovery from drug effects was not examined closely, but in 3 out of 3 cells examined there was recovery of the noxious heat response to within 50-85% of control at 6-15 min after terminating ionophoresis of the drug. The inset shows the position of the cutaneous receptive field on the ipsilateral hind limb.


Controls
112
96
80
64
48
32
16
0
Counts per 400ms
Brush
Pinch
30°C 48° 48° 30°
Noxious heat
L-659874, 15nA for 9min
112
96
80
64
48
32
16
0
Brush
Pinch
30°C 48° 48° 30°
Noxious heat
10 s

the mean pre-drug control value it was calculated that 10nA of L-659,874 caused a 49% inhibition of the responses to noxious heat in responsive cells (see Figure 2.7) with no detectable effect in noxious pinch (99 ± 8% of pre-drug control), innocuous brush (103 ± 9% of control) or spontaneous activity (93 ± 7% of pre-drug activity). At a higher ejection current of 20nA, the selective inhibition of noxious heat response was seen in all cells tested (9 out of 9) and this was of a greater degree (mean 66% inhibition), than the lower ejection current. Again there was no significant change in the responses to noxious pinch (85 ± 11%), innocuous brush (104 ± 6%) or spontaneous activity (95 ± 12% of pre-drug activity). In 4 cases, where both currents were tested on the same cell this apparent dose-dependence was particularly clear.

In 3 neurons where recovery was investigated, the inhibition of heat responses by L-659,874 had partially recovered (to 50-85% of controls) within 6-15 minutes after cessation of ionophoresis. In the majority of cells tested here, responses to noxious heat declined rapidly on cessation of the stimulus (to less than 20% of the response during the stimulus, within the subsequent 10 seconds). For each of the drugs tested there were, however, examples of relatively prolonged after-discharges, retaining around 50% of the peak activity during the first 10 second post-stimulus period. There was no evidence that effects of any of the drugs were greater (or in any way different) in post-stimulus periods than during application of the heat stimulus (Figure 2.8).

2.4.5 Effects of Ionophoretically Applied Neurokinin Agonists

The effects of ionophoretically applied NK_1 and NK_2 receptor agonists on multireceptive dorsal horn neurons were assessed as previously described. Control responses were tested and then the drug under study was ionophoresed for 1 minute before the sensory responses were tested.

(a) Ionophoretically Applied NK_1 Agonist

The highly-selective NK_1 receptor agonist [N-acetyl-Arg^6,Sar^9Met $(O_2)^{11}$]substance P-(6-11) (Regoli *et al,* 1988) was tested on neuronal responses to cycled cutaneous stimuli either at low (31 ± 4nA) or higher (57 ± 5nA) ionophoretic currents.

Ionophoresis of [N-acetyl-Arg^6, Sar^9Met $(O_2)^{11}$]substance P-(6-11)

The effects of the highly-selective NK_1 receptor agonist [N-acetyl-Arg^6, $Sar^9Met(O_2)^{11}$]substance P-(6-11) were investigated in 13 multireceptive neurons

in laminae III-V of the spinal dorsal horn. When ejected at a low current range, there generally was no effect on the spontaneous activity in the neurons examined. There was a very slight increase in the basal activity to 121 ± 13 % of pre-drug control when ejected at 31 ± 4nA for a mean time of 5 ± 1 minutes ($P>0.05$ Wilcoxon Test).

However, in 9/13 neurons tested, when this NK_1 agonist was ejected at 31 ± 4nA there was a marked inhibition of the activity evoked by innocuous brush in comparison with pre-drug controls (to 52 ± 5% of mean pre-drug control). (The results were analysed off-line and taking spontaneous activity into account the values were calculated as a percentage of mean pre-drug control value.) There was no significant change to either noxious pinch or noxious heat responses after ionophoresis of low currents of the NK_1: the noxious heat response was 98 ± 5% of control and likewise the noxious pinch response was 92 ± 6% of the mean pre-drug control. The summary, Figure 2.7 shows that whereas the responses to spontaneous activity and noxious pinch and heat were not significantly different from control values ($P>0.05$), the response to innocuous brush was significantly decreased from mean pre-drug control ($P>0.05$).

Six neurons were additionally tested with a higher current range and showed similar results to the low current range in 6 out of 6 neurons tested over a mean ejection current of 57 ± 5nA for 6 ± 2 minutes of [N-acetyl-Arg^6, Sar^{9}Met $(O_2)^{11}$]substance P-(6-11). There was no significant change in either noxious pinch or noxious heat (91 ± 14% and 106 ± 11% of mean pre-drug control respectively) but in an analogous manner to the lower dose range, there was a significant decrease in the response to innocuous brush. The higher ejection current, nevertheless, did cause a marked increase in the spontaneous activity to 536 ± 88% of the control level. Current and vehicle control tests were carried out and were found not to replicate the drug effect in any way.

(b) Ionophoretically-Applied NK_2 Agonists

Two NK_2 agonists were tested in these studies, the endogenous ligand neurokinin A (NKA) and the highly-selective agonist at NK_2 receptors, GR 64349 (Hagan *et al,* 1991). Both agonists elicited similar responses when ejected at higher currents onto the multireceptive dorsal horn neurons.

Ionophoresis of Neurokinin A

The effect of ionophoresis of neurokinin A was investigated in a total of 8 multireceptive neurons in laminae III-V of the spinal dorsal horn. Two dose

ranges were investigated, the lower range had an ejection current of 11 ± 5nA and was maintained for 7 ± 1 minutes (mean ± s.e.m.). At this low ejection current there was no significant change in the level of spontaneous activity compared to control levels (112 ± 10% of mean pre-drug control). There was however a selective facilitation of the responses to noxious thermal stimuli; in 6/8 cells tested there was a facilitation to 155 ± 13% of mean pre-drug control (mean ± s.e.m., $P<0.05$). The responses to innocuous brush and noxious pinch remained essentially unaltered (89 ± 11% and 108 ± 10% of pre-drug controls respectively) and although one cell showed a 30% increase in noxious pinch response, this was not reproduced in the other examples. The ongoing firing frequency record shown in Figure 2.11 shows the typical effect of low ejection currents of the NK_2 agonist NKA on the spontaneous activity and cutaneous sensory responses.

When ejected over a higher current range of 44 ± 4nA for a mean time of 6 ± 1 minutes, the outcome was a different set of influences on spontaneous activity and sensory responses in comparison with the lower ejection current range. The spontaneous activity was significantly increased in a consistent manner to 354 ± 61% of the pre-drug spontaneous activity ($P<0.05$) in 6 out of 8 cases. However, when corrected for the elevated baseline, it was seen that noxious heat responses were attenuated to 64 ± 16% of the mean pre-drug control values whilst the responses to noxious pinch and innocuous brush were essentially unaltered (79 ± 19% and 120 ± 21% of pre-drug control values respectively; no significant changes). After ionophoresis of NKA, in 2 cases the increment in spontaneous activity was equivalent to the magnitude of reduction in heat responses. However, in all other cases this NKA-induced reduction in heat responses was much greater than the actual increase in spontaneous activity. Recovery was observed within 6-12 minutes after terminating ionophoresis and none of the effects were reproduced in current control tests. In 4 cells where [N-acetyl-Arg^6, $Sar^9Met(O_2)^{11}$]substance P-(6-11) and NKA were each tested, there were clear excitatory responses to both NK_1 and NK_2 receptor agonists.

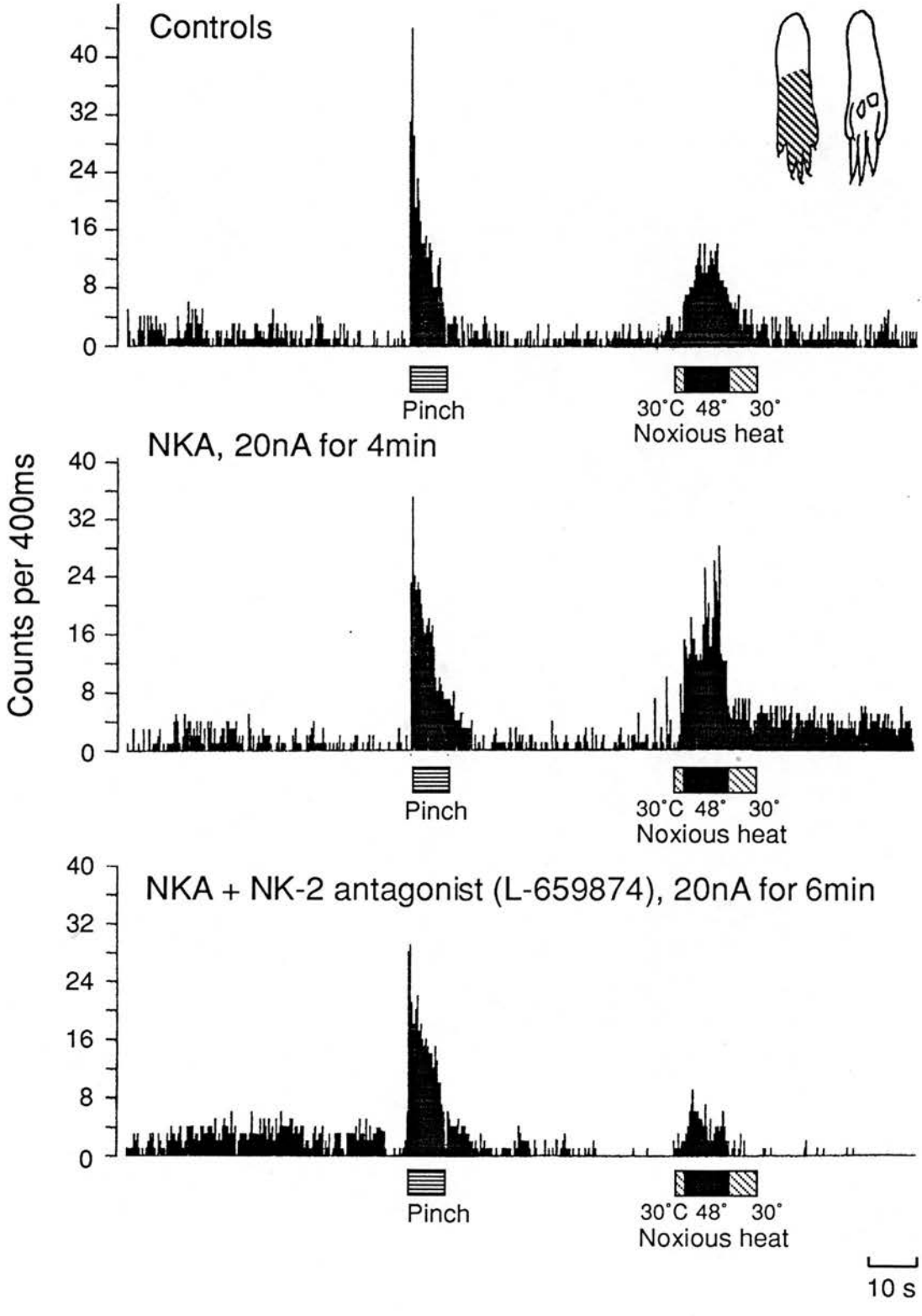
Ionophoresis of GR 64349

Ionophoresis of the selective NK_2 agonist GR 64349 was studied in 5 multireceptive laminae III-V dorsal horn neurons. At a mean ejection current of 66 ± 5nA, the agonist produced similar results to the higher dose range of the endogenous ligand NKA. When the agonist was ejected for a mean time of 7 ± 1 minute, spontaneous activity was clearly increased from baseline levels to 398 ± 54% of the mean pre-drug control ($P<0.05$, Wilcoxon Test). Innocuous brush

Figure 2.11

Ongoing firing frequency records showing typical effects of the NK_2 receptor agonist neurokinin A in the absence or presence of the NK_2 receptor antagonist L-659,874 on the somatosensory responses of a laminae III-V neuron.

Neurokinin A caused a selective facilitation of responses to thermal but not mechanical noxious stimuli. Responses to innocuous brush (not shown here) were also unaltered (see Figure 2.7). Results were typical of 6 neurons tested with low currents of neurokinin A. After the middle trace had been recorded, iontophoresis of L-659,874 was begun in addition to neurokinin A. A marked attenuation of the neurokinin A-amplified response to noxious heat to below control levels was observed in all 3 cells so tested. The inset shows the position of the cutaneous receptive field on the ipsilateral hind limb.


Controls
40
32
24
16
8
0
Pinch
30°C 48° 30°
Noxious heat
NKA, 20nA for 4min
Counts per 400ms
NKA + NK-2 antagonist (L-659874), 20nA for 6min
10 s

and noxious pinch responses were essentially unaltered (111 ± 13 and 88 ± 20% of mean pre-drug control), the noxious heat response, when normalised for the elevated baseline, was diminished to 75 ± 11% of control values ($P>0.05$). Recovery of the spontaneous activity and the heat response was observed within 6 to 12 minutes of terminating ionophoresis; the responses returning close to baseline levels.

2.4.6 Co-Ionophoresis of Both NK_1 Agonist and Antagonist.

The response to co-administration of both NK_1 agonist and antagonist was assessed in 3 laminae III-V multireceptive dorsal horn neurons. Initial ejection of low currents of the highly selective NK_1 agonist [N-acetyl-Arg^6, $Sar^9Met\ (O_2)^{11}$] substance P-(6-11) resulted in responses typical of the 13 cells examined in total; there was a clear inhibition of the response to innocuous brush with no discernible effect on either spontaneous activity or to noxious pinch and heat responses. Once a stable response to the agonist had been achieved, co-ionophoresis with the specific NK_1 antagonist GR 82334 was initiated. In 3 out of 3 neurons, ionophoresis of GR 82334 reversed the [N-acetyl-Arg^6, $Sar^9Met\ (O_2)^{11}$]substance P-(6-11) - induced decrease in brush responses, with no significant effect on the noxious pinch and heat responses. The effect of GR 82334 on the increase in spontaneous activity brought about by high ejection currents of [N-acetyl-Arg^6, $Sar^9Met\ (O_2)^{11}$] substance P-(6-11) was not investigated in this series of experiments.

2.4.7 Co-Ionophoresis of Both NK_2 Agonist and Antagonist.

Three multireceptive dorsal horn laminae III-V neurons were used to investigate the action of co-administration of both NK_2 agonist and antagonist by an ionophoretic route. The response to a low ejection current of NKA was determined and a further 3 out of 3 cells where there was a selective facilitation of the thermal nociceptive responses, with no apparent effect on spontaneous activity, noxious pinch or innocuous brush. This response is typical of the neurons tested with low currents of NKA,(see Figure 2.11). Once a strong and stable response to the NK_2 agonist had been achieved, co-ionophoresis with the highly selective-NK_2 antagonist L-659,874 was initiated. Figure 2.11 demonstrates the typical effects achieved with first, low currents of NKA followed by a co-ionophoresis with the NK_2 antagonist L-659,874. The NKA induced facilitation of the noxious heat response was markedly attenuated to below control levels after co-ionophoresis with L-659,874. This is consistent with reversal of the effect of

exogenous NKA by the selective NK_2 receptor antagonist but cannot be unequivocal, since the antagonist alone had inhibitory effects on thermal nociceptive responses (Figure 2.10). The effect of L-659,874 on the increase in spontaneous activity brought about by high ejection currents of NKA was not investigated in the present study.

2.5 DISCUSSION

Multireceptive dorsal horn neurons from laminae III-V of the rat dorsal horn spinal cord were identified using both depth measurements (taken from the microdrive) in combination with Pontamine Sky Blue histology. Mulitreceptive neurons were chosen to be studied as they can integrate both nociceptive and non-nociceptive information (Iggo, 1974; Handwerker *et al*, 1975) and allow ready comparison of the processing of these inputs. Ionophoresis of the direct excitant D,L-homocysteic acid (DLH) at the same currents used for ionophoresis of agonists/antagonists caused reproducible excitation throughout each experiment, reducing the possibility that recordings were from primary afferent fibres (Goodchild *et al*, 1982) or that any drug responses were due to movement of the recording electrode away from or closer to the recording site. Current and vehicle controls were routinely carried out using ejection of NaCl and DMF (0.3% dimethylformamide in water) respectively and were unable to reproduce any of the observed drug actions described below.

Although SP is known to cause excitation of nociceptive dorsal horn neurons (Henry, 1976; Randic and Miletic, 1977; Zieglgansberger and Tulloch, 1979), these experiments provided no clear evidence that NK_1 receptors participate in transducing brief nociceptive inputs to laminae III-V neurons of rat spinal dorsal horn under the present conditions. The highly selective NK_1 antagonist, L-668,169 was used. However, as its potency is reported to be reduced in rodent bioassays compared to those in other species (Pattachini *et al*, 1992), two other NK_1 antagonists were also tested; GR 82334 which retains high potency at rat NK_1 receptors (Beresford *et al*, 1992) and [D-Pro^4,D-$Trp^{7,9,10}$,Phe^{11}] substance P-(4-11), which is effectively antinociceptive in the mouse formalin paw model (Murray *et al*, 1991). From the results it can be seen that ionophoretic application of GR 82334, L-668,169 or [D-Pro^4,D-$Trp^{7,9,10}$,Phe^{11}] substance P-(4-11), to laminae III-V dorsal horn neurons, at pharmacologically effective currents, induced no significant effects on spontaneous activity nor on the responses to noxious pinch and noxious heat. In

contrast, each NK_1 receptor antagonist caused a modest facilitation of their responses to innocuous brush in the majority of cells. As ionophoresis of the NK_1 receptor antagonists regularly facilitated the responses to innocuous cutaneous stimuli, this suggests that their lack of effect on nociceptive responses was not due to failure to reach the relevant sites at the concentrations used, nor for technical reasons. Further support that this effect of L-668,169, GR 82334 and [D-Pro^4,D-Trp^{7,9,10},Phe^{11}]substance P-(4-11) on non-nociceptive responses was due to NK_1 receptor antagonism is strengthened by the observation that the NK_1 antagonists used are all structurally quite distinct compounds but yet ionophoresis of low currents of each has similar consequences. These observations are substantiated by the precisely inverse results obtained with the highly selective NK_1 agonist [N-acetyl-Arg^6,Sar^{9}Met$(O_2)^{11}$substance P-(6-11). At low ejection currents, ionophoresis of [N-acetyl-Arg^6,Sar^{9}Met$(O_2)^{11}$substance P-(6-11) to rat laminae III-V dorsal horn neurons, generally induced no significant effects on their spontaneous activity nor on the responses to noxious cutaneous stimulation, but there was a marked inhibition of the activity evoked by innocuous brush in comparison to pre-drug controls. However, when ionophoresed at a higher current range, [N-acetyl-Arg^6,Sar^{9}Met$(O_2)^{11}$substance P-(6-11) likewise did not affect the responses to noxious cutaneous stimulation but in an analogous manner to the lower dose range, significantly decreased the response to innocuous brush. The higher ejection current nevertheless, did cause a marked increase in the spontaneous activity consistent with the possibility of a distinct population of NK_1 receptors causing relatively direct excitation of laminae III/V neurons, in addition to the population which appears to modulate their non-nociceptive inputs. Nevertheless, it is possible that the antagonists have unknown side effects or that more complex interactions are occurring.

Co-ionophoresis of both NK_1 receptor-selective agonist and antagonist further strengthens the possibility that NK_1 receptors are involved selectively in non-nociceptive responses. Once a stable response to the agonist had been achieved, co-ionophoresis with the specific NK_1 antagonist was initiated. The results show that GR 82334 reversed the [N-acetyl-Arg^6,Sar^9,Met$(O_2)^{11}$] substance P-(6-11)-induced selective decrease in brush response.

The observations that ionophoretic administration of NK_1 receptor antagonists selectively facilitate the response to innocuous brush (with no significant effect on responses to noxious pinch and heat), suggests that NK_1 receptors are responsible for an attenuating influence on non-nociceptive inputs to these cells and that the antagonist is acting to reverse the action of the endogenous

receptor agonist, substance P, which has been released during the course of our experimental procedure. Although the factors responsible for substance P release are unclear, it does not appear to be responsible for acutely mediating the increased activity elicited by brief thermal or mechanical noxious stimuli. This data is entirely consistent with previous experiments carried out using cats (Fleetwood-Walker *et al,* 1990) and in agreement, other groups have reported a similar lack of involvement of NK_1 receptors in mediating brief nociceptive inputs (both thermal and mechanical) to deeper dorsal horn neurons (Duggan *et al,* 1979; Willcockson *et al,* 1984). However, these effects are at variance with the results of others. It has been reported that administration of the non-peptide NK_1 antagonist CP-96,345 results in inhibition of responses of nociceptive dorsal horn neurons to C-fibre strength electrical simulation or of the after-discharge responses to brief noxious pinch and heat stimuli (De Koninck and Henry, 1991; Radhakrishnan and Henry, 1991). There was little effect on the initial phase of excitatory responses to brief heat stimuli and inconsistent effects on pinch-evoked excitation. It is possible that CP-96,345 is effective only on late components of nociceptive responses, however the use of an extremely profound pinch stimulus as well as evidence that CP-96,345 is a potent antagonist of 'L'-type Ca^{2+} channels (Schmidt *et al,* 1992) and furthermore that such compounds are effective antinociceptive agents (Miranda *et al*, 1992) suggest that actions of this compound other that on NK_1 receptors may be involved. Although both peptide and non-peptide NK_1 receptor antagonists have been reported to block the late prolonged but not the brief excitatory postsynaptic potentials induced in dorsal horn neurons by stimulation of afferents at C-fibre strength or by noxious stimuli (Urban and Randic, 1984; De Koninck and Henry, 1991), in our experiments, there was no evidence that NK_1 receptor antagonists were any more effective in the 20s of after discharge following a noxious heat stimulus than they were during the response itself (Figure 2.8).

In contrast to the results concerning NK_1 receptors, the highly selective NK_2 receptor antagonist L-659,874 caused a marked and highly consistent inhibition of thermal nociceptive responses without affecting spontaneous activity or responses to innocuous brush and noxious pinch. Inhibition by the NK_2 receptor antagonist showed a striking selectivity for thermal rather than mechanical nociceptive stimuli, a result also noted in cat with the moderately selective NK_2 receptor antagonist [D-Pro^4,Lys^6,D-$Trp^{7,9,10}$,Phe^{11}] substance P -(4-11) (Fleetwood-Walker *et al*, 1990).

Consistent with a role of an NK_2 receptor agonist such as neurokinin A in thermal, but not mechanical nociception, ionophoretic administration of NKA to laminae III-V dorsal horn neurons caused a selective facilitation of thermal nociceptive responses with no significant effect on background (spontaneous) activity, or responses to innocuous brush and noxious pinch (Figure 2.11), a result also seen in cat with neurokinin A and other agonists with NK_2 receptor selectivity (Fleetwood-Walker *et al*, 1990)). Although NKA is the preferred ligand for the NK_2 receptor, it also shows a slight affinity for both NK_1 and NK_3 receptors present in spinal cord tissue (see Maggi *et al*, 1993). However, the NK_2 receptor selective agonist GR 64349 was also used in this set of experiments and administration of this agonist resulted in an equivalent selective facilitation of thermal nociceptive responses. Further substantiating that NK_2 receptors are selectively involved in thermal nociceptive responses is the observation that the selective NKA-induced facilitation of the response to noxious heat is markedly attenuated when both NKA and the selective NK_2 receptor antagonist L-659,874 are co-ionophoresed.

In the current experiments, both neurokinin A and GR 64349 further caused marked and consistent increases in spontaneous activity of laminae III-V dorsal horn neurons, consistent with the idea that NK_2 receptor activation is an important element in the transduction of thermal nociceptive inputs. It is of course possible that co-released transmitters may also subserve an important role. Interestingly, although both NK_1 and NK_2 receptor agonists increase the spontaneous activity in laminae III-V cells, the situation is quite different in lamina I where only NK_2 but not NK_1 receptor agonists are effective (Fleetwood-Walker *et al*, 1992).

These results would thus appear to suggest that for these tachykinin receptors, it is actually the NK_2 receptor which plays an important role in mediating brief thermal noxious responses within laminae III-V of the spinal dorsal horn. The mechanistic basis for this is unclear, since polymodal C-afferents are reported to contain tachykinins, (Leah *et al*, 1985) and are likely to contribute greatly to both responses. However, subpopulations specifically sensitive to either thermal or mechanical nociceptive stimuli may exist (Perl, 1984; Besson and Chaouch, 1987; Treede *et al*, 1992). Small dorsal root ganglion neurons are very heterogenous in terms of their neuropeptide content (Leah *et al*, 1985; O'Brien *et al*, 1989; Levine *et al*, 1993) and there is evidence that thermal/mechanical noxious stimuli can differentially elicit release of neuropeptides into spinal perfusates (Duggan *et al*, 1987, 1988; Kuraishi *et al*, 1989; Tiseo *et al*, 1990).

Although SP and NKA originate from a single gene, differential RNA processing can result in mature transcripts either encoding or lacking the NKA sequence (Nawa *et al*, 1984; Krause *et al*, 1987). This may be a cell-specific phenomenon occurring differentially in certain functional sub-populations of small dorsal root ganglion cells. Substance P release in the spinal cord has been reported following noxious pinch, intraplantar formalin, topical methylene chloride and flexion of a kaolin/carrageenan knee-joint (Duggan *et al*, 1988; Kuraishi *et al*, 1989; Schaible *et al*, 1990; McCarson and Goldstein, 1991). Thermal cutaneous stimuli, however, were only effective at damaging skin temperatures considered to result in irreversible inflammatory cutaneous lesions (Go and Yaksh, 1987; Duggan *et al*, 1988; Kuraishi *et al*, 1989). SP release was localised to a discrete focal area of the SG (Duggan *et al*, 1988) where it is degraded rapidly (Duggan *et al*, 1992). In contrast, both noxious mechanical stimuli and noxious thermal stimuli (at skin temperatures below those producing inflammatory damage), but not innocuous stimuli, were effective in releasing neurokinin A (Duggan *et al*, 1990) which persisted and spread diffusely thoughout the dorsal horn. Furthermore, the selective NK_2 antagonist MEN 10376 was a potent antagonist of capsaicin-evoked C fibre excitation of dorsal horn neurons (Urban *et al*, 1992; Nagy *et al*, 1993), whereas the NK_1 receptor antagonist CP-96,345 did not inhibit capsaicin-evoked responses (Nagy *et al*, 1993). As capsaicin is believed to selectively activate polymodal nociceptors (Fitzgerald, 1983), these results suggest that activation of dorsal horn neurons by polymodal nociceptors involve mainly NK_2 rather than NK_1 receptors. Also, Xu *et al*, (1991) described that the NK_2 receptor antagonist, [Tyr^5,D-$Trp^{6,8,9}$,Arg^{10}]neurokinin A-(4-10) (MEN 10207) selectively reversed the facilitation by neurokinin A to the sural nerve of the spinal nociceptive flexor reflex. In agreement, the NK_2 receptor selective antagonist MEN 10376 inhibited the ventral root potentials (VRPs) evoked by C fibre strength electrical stimulation of the ipsilateral dorsal roots, whereas the NK_1 receptor antagonists RP 67580 and CP-96,345 were ineffective (Thompson *et al*, 1993). However, in a condition of UV-induced hyperalgesia, the facilitated VRPs were significantly reduced by both NK_1 and NK_2 receptor antagonists (Thompson *et al*, 1993) suggesting that NK_1 receptors are of greater importance in mediating responses to noxious stimuli in more prolonged pain states. Correspondingly, only the facilitation of nociceptive flexor reflex by substance P or C-afferent conditioning stimuli and conditioning stimulation of the gastrocnemius nerve (and not the reflex itself) was inhibited by the NK_1 receptor antagonist spantide II (Wiesenfeld-Hallin *et al*, 1990).

The data from behavioural experiments also point towards the involvement of substance P in mediating inflammation-supported nociception rather than responses to brief noxious stimuli. In acute behavioural nociceptive studies, the evidence for involvement of NK_1 receptors is poor. Although some groups have demonstrated a reduction in hot-plate and paw-pressure thresholds following intrathecal administration of SP or a selective NK_1 agonist (Post and Folkers, 1985; Cridland and Henry, 1986,1988; Picard *et al*, 1993; Yashpal *et al*, 1993), others demonstrated antinociception (Doi and Jurna, 1981) or evidence suggesting that NK_1 receptor plays no role in behavioural nociceptive responses (Piercey *et al*, 1981; Gamse and Saria, 1986; Elliott *et al*, 1992; Garces *et al*, 1992). However, behavioural reports indicate that NK_1 receptors are of greater importance in mediating responses to noxious stimuli in more prolonged pain states. In a tonic nociceptive test, pre-treatment with the selective NK_1 antagonists CP 99994 (Elliott *et al*, 1992) and CP-96,345 (Yamamoto and Yaksh, 1991; Birch *et al*, 1992; Yashpal *et al*, 1993) significantly reduced the second (inflammation-supported) phase of the formalin-induced response to intraplantar formalin. Similarly, the racemic mixture of CP-96,345 has shown antinocieptive and anti-oedema activity in carrageenan-induced hyperalgesia (Birch *et al*, 1992) and mustard oil-induced foot oedema (Lembeck *et al*, 1992) in rats as well as in acetic acid writhing in mice (Nagahisi *et al*, 1992).

Thus these results provide evidence for the involvement of NK_2 but not NK_1 receptors in acute nociceptive transmission. However, the evidence is accumulating that NK_1 receptors are of greater importance in mediating responses to noxious stimuli in more prolonged pain states. Therefore it was decided to investigate the involvement of NK_1 and NK_2 receptors in sustained nociceptive states.

CHAPTER 3:
The Effects of Neurokinin Antagonists on Mustard Oil-Evoked Activation of Rat Dorsal Horn Neurons.

3.1 AIMS

The present experiments were designed to compare the contribution of NK_1 and NK_2 receptors in dorsal horn to the sustained neuronal activity induced by the peripheral application of the chemical algogen mustard oil (reported to be a selective activator of C afferents).

3.2 MATERIALS

Animals: Male Wistar rats were obtained from Charles River UK Ltd, Margate, Kent, UK.

Anaesthetics: Alpha-chloralose and urethane were obtained from Sigma Chemical Company, Poole, Dorset, UK; Halothane (flurothane) was obtained from ICI Pharmaceuticals, Macclesfield, Cheshire, UK.

Laboratory Chemicals: Standard laboratory chemicals were of Analar grade and from the following suppliers: Sodium chloride (NaCl), DLH (D, L, homocysteic acid) and Pontamine Sky Blue (PSB) were obtained from Sigma Chemical Company, Poole, Dorset, UK; allyl-isothiocyanate (mustard oil) was purchased from Aldrich Chemical Company Ltd, Gillingham, Dorset, UK; Halothane (Flurothane) was obtained from ICI Pharmaceuticals, Macclesfield, Cheshire, UK. Agar was obtained from Oxoid Ltd, Basingstoke, Hampshire, UK and paraffin was purchased from Scotlab, Lanarkshire, UK.

Drugs: The tachykinin antagonists, acetyl-Leu,Met,Gln,Trp,Phe-NH_2 (L-659,874) and cyclo(Gln,D-Trp,Me-Phe,(R)Gly[ANC-2]Leu,Met)$_2$, (L-668,169) were obtained from Cambridge Research Biochemicals & [D-Pro^9[spiro-γ-lactam]-Leu^{10},Trp^{11}]physalaemin-(1-11), (GR 82334) was a gift from Glaxo Group Research; 2-[1-imino-2-(2-methoxyphenyl)ethyl]-7,7 diphenyl-4 perhydroisoindolone (RP 67580) was a gift from Dr C Garret, Rhone-Poulenc Recherché-Development, France and (S)-N-methyl-N[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichloro phenyl)butyl] benzamide (SR 48968) a gift from Dr X Emonds-Alt, Sanofi Recherché, France.

3.3 METHODS

3.3.1 General Methods

See Chapter 2 for details of animals, surgical techniques, histological determination of laminar location and general procedures for recording & ionophoresis. Extracellular recordings from single laminae III-V dorsal horn neurons, were made through the central barrel of a 7-barrelled microelectrode containing 4M NaCl, pH 4.0-4.5. Drugs were ionophoresed from the side barrels. One side barrel contained 1M NaCl (pH 4.5-5.0), for automatic current balancing, another barrel was filled with PSB for marking the position of recording sites and the other electrode barrels contained various combinations of the following selective NK_1 and NK_2 antagonists:- L-659,874; L-668,196 and L-659,877 (0.1mM in 0.3% DMF, pH 4.0-4.5); GR 82334 (1mM in distilled H_20, pH 4.0-4.5); R396 (0.1mM in 0.3% DMF, pH 4.0-4.5); RP 67580 and SR 48968 (1mM in distilled H_20, pH 4.0-4.5).

3.3.2 Application of Mustard Oil and Quantification of Drug Responses

All of the neurons recorded had cutaneous receptive fields on the ipsilateral hindlimb which were multireceptive. Neurons were initially located by their responsiveness to widespread manual brushing of the ipsilateral hindlimb and cutaneous fields localised as described in Chapter 2. However, only neurons which had a robust response to noxious heat were used, as these were more susceptible to showing a prolonged increase in firing rate upon application of mustard oil. The receptive field of each neuron was carefully mapped out on the ipsilateral limb using a marker pen. Mustard oil (3-isothiocyanato-prop-1-ene) was diluted to 5-20% (usually 8%) in paraffin oil and applied to the receptive field area using a paintbrush. This procedure was repeated every 5 minutes until a sufficient increase in discharge was maintained over a substantial period of time (over tens of minutes). The mustard oil could influence the neurons in one of two ways, either firstly with an immediate increase in discharge upon cutaneous application which remained sustained; or secondly it took several applications of the algogen to increase the firing rate. Drugs were tested only after mustard oil-evoked activity was stable and had been continuous for at least 1-2 minutes. The effect of drugs on the mustard oil induced activity was tested and in these studies, the ionophoretic currents of the antagonists were increased stepwise from 10-80nA. Each drug was ejected for 1-2 minutes unless excitation occurred, in which case, the drug was switched off and the neuron allowed to recover fully before the

next drug. At the end of each experiment, Pontamine Sky Blue was ejected to mark recording sites for identification by histology as described earlier.

3.3.3 Analysis

Before the application of mustard oil, the cutaneous sensory responses were analysed as described in Chapter 2. The mustard oil-increased firing rate was also recorded on-line by computer (IBM PS/2-70-121) and the percentage increase in activity after mustard oil application was analysed over a three minute period, from a section of data in which the activity of the cell had reached a stable level. This was achieved by integrating the number of action potentials in 5 second intervals every 20 seconds throughout the 3 minute period. The percentage inhibition of firing caused by antagonists was analysed by integrating the number of action potentials in a 60 second period, immediately before and following the administration of antagonists.

3.4 RESULTS

3.4.1 Characteristics and Receptive Field Properties of Neurons

The present results were obtained from 35 neurons located in laminae III-V in the dorsal horn of 33 rats. Laminar location was estimated as the depth recording on the microdrive which for laminae III-V was 250-600μm. Very occasionally recording took place from cells located slightly more superficially to this depth but recording from cells found deeper than 600μm was only occasional since there appeared to be a tendency for unresponsiveness below this depth. Upon cessation of recording, each cell was marked by ejection of Pontamine Sky Blue (PSB) for 10-15 minutes and at the end of the experiment, the spinal cord was removed and histologically treated to enable identification of the recording electrode position on transverse sections of the cord (see Figure 2.5).

All the neurons studied had cutaneous receptive fields located on the hairy side of the foot and toes or the flank of the ipsilateral hindlimb. It was important that the receptive field of the neuron was not located on the glabrous skin of the foot as poor penetration by mustard oil resulted in the neuron under examination not being activated by the algogen. The neurons were initially identified by widespread manual brushing over the ipsilateral hindlimb and foot; the receptive field was then mapped with an innocuous brush stimulus and the receptive field area marked with waterproof ink. The size of the receptive field area varied considerably between the neurons, from as small as a single digit to the whole

flank of the ipsilateral limb and lower body; throughout the experiment the receptive field size was continually monitored and any changes were noted.

All the neurons were multireceptive (responded to both innocuous and noxious stimuli). It was particularly important that the neuron had a robust response to noxious heat, as any neurons tested without this were generally not activated by mustard oil. The noxious heat response is derived mainly from the C fibres and evidence has been presented that mustard oil elicits activation of largely C afferents (Heapy *et al,* 1987; Woolf and Wall, 1986), although it is possible that some Aδ activation also occurs transiently (Harris and Ryall, 1988). Generally before application of the mustard oil, the background activity of the neurons examined was very low (less than 1Hz). If the background activity was too high before application of mustard oil, then it masked the facilitatory response and so these neurons were not used.

In some experiments, ionophoresis of D, L-homocysteic acid (DLH) was carried out to ensure that any apparent changes were not due to movement of the recording electrode relative to the neuron in that excitatory responses to this agent remained relatively constant. The response to ionophoresis (at the same current) of DLH remained constant throughout the experiment.

3.4.2 Control Responses

Neurons under consideration were tested with NaCl current controls. They were examined under identical conditions to the antagonists under study, with ejection for at least 1 minute, increasing the current in a 10-20 nA step-wise manner every consecutive minute. In 2 out of 2 neurons tested at up to 80nA, there was no effect after ionophoresis of NaCl, whereas L-659,874 promptly caused greater than 50% inhibition of evoked-activity. See Figure 3.2a for a typical example.

3.4.3 Cutaneous Application of Mustard Oil

Following mustard oil (allyl isothiocyanate) application to the receptive field area, 34 out of 35 multireceptive laminae III-V dorsal horn neurons had a significant and large increase in activity with firing rates of 138 ± 33 fold of background (mean ± s.e.m.). In the majority of cases examined (28 out of 35 cells), this increased activity was consistently maintained for at least 28 minutes.

There were two general patterns which the increased mustard oil activity followed; some cells had an immediate increase in activity after one application of mustard oil which remained sustained for at least 7 minutes (Figures 3.1, 3.2b and

3.3a). However, in most cases (n=22), the steady elevated firing rate was only achieved by repeated application of mustard oil (3-5 times to the same site over a period of 10-15 minutes), see Figure 3.4. This apparent different sensitivity to mustard oil had no obvious effects on the pharmacological results obtained.

Records such as those shown in Figure 3.2 were analysed off-line to calculate the percentage inhibition by the tachykinin antagonists of the mustard oil evoked baseline. Before analysis it was important to ensure that the elevated mustard oil-induced activity had remained at a constant level for at least 2 minutes. The number of mustard oil- induced action potentials in 3 separate 30 second periods were integrated and an average value calculated (taking spontaneous activity prior to mustard oil activity into account). After ionophoresis of the drug under study, a second average value was calculated and this was expressed as a percentage of the mean pre-mustard oil control value. Statistical significance of drug effects was calculated using the Mann Whitney U-test. Where recovery was observed, the results were calculated as a percentage of the initial mustard oil activity.

3.4.4 Mustard Oil-Induced Peripheral Effects

After cutaneous application of mustard oil, over the course of the experiment, the receptive field area became pink and slightly swollen. After recording from one neuron on the ipsilateral side of the cord, this side of the animal was not used again even if the cell did not respond to peripheral application of mustard oil. Care was taken to ensure that the mustard oil was not transferred to the contralateral limb by wrapping the limb in waterproof tape after each application. No peripheral effects due to mustard oil application were observed on the contralateral side.

3.4.5 Effect NK_2 Antagonists on Mustard Oil Induced Activity.

The effect of NK_2 antagonists on the mustard oil-evoked activity was investigated using two methods of administration.

(a) Effect of Ionophoretically Applied NK_2 Antagonists.

The drugs were tested only after the mustard oil-elevated activity was stable and had been continuous for at least 1-2 minutes. When the highly-selective peptide NK_2 antagonist, L-659,874, was ionophoresed at 10-80nA, in 23 out of 24 cells tested there was a clear and relatively maintained inhibition of mustard oil-evoked activity (see Figures 3.1, 3.2 and 3.3) to 50 ± 7% (mean ± s.e.m.) of prior

control activity ($P<0.05$, Mann-Whitney U-test). Inhibition began after an onset delay of 0-120 seconds (mean = 38 ± 9 seconds, n=23). In 19 cases where currents of ≥ 40nA were tested, clear inhibition was always apparent within 30 seconds. Recovery was observed in 13 out of 17 cases examined after terminating ionophoresis of L-659,874 (Figure 3.2a) and in about half of these cases (n=6), inhibition was maintained at its maximal level for no more than 30-60 seconds (mean = 48 ± 6 seconds) even in the continued presence of L-659,874 (Figures 3.2b and 3.3a). Increasing the ejection currents however, produced a more marked inhibition in 15 out of 19 neurons tested, with a typical example showing 17, 33 and 85% inhibition at 20, 30 and 40nA respectively (see Figure 3.2b). Similar results were achieved when using the highly selective non-peptide antagonist SR 48968. Figure 3.5b shows a typical example of one of the 3/4 cells tested with ionophoretic currents of 15-70nA, there was a similar pattern of sustained inhibition of the mustard oil-induced firing rate to 52.8 ± 7.8 % ($P<0.05$, Mann-Whitney U-test) of prior control activity. Recovery was observed in each of the cases tested.

Analysis was carried out off-line and the effect of L-659,874 on the mustard oil-evoked activity was calculated for each 1 minute ionophoresis period. The number of spikes integrated into each 1 minute ionophoresis period were expressed as a percentage of the pre-drug mustard oil-evoked activity.

Recovery was assessed every minute after the termination of ionophoresis. The number of spikes integrated in each 30 second period were calculated and expressed as a percentage of pre-mustard oil- evoked activity. Once the neuron had sufficiently recovered, the next drug was ionophoresed. Statistical significance of drug effects was calculated using the Mann Whitney U-test .

(b) Effect of Intravenous Application of NK_2 Antagonists

The non-peptide NK_2 antagonist SR 48968 (Emonds-Alt *et al,* 1992), when intravenously injected, gave entirely similar results to ionophoresis of the peptide drug L-659,874. Figure 3.5d demonstrates when SR 48968 was intravenously injected at a dose of 1mg/kg and in 5/5 neurons tested there was a marked decrease in the mustard oil-induced firing rate to 38.1 ± 3.1% of pre-drug control levels (mean ± s.e.m.) ($P<0.05$, Mann-Whitney U-test). The maximum inhibition of mustard oil-evoked activity usually occurred 2-3 minutes after intravenous administration of the non-peptide drug and full recovery was observed in only 2 out of the 5 cells examined after 5-12 minutes, however in a further 2

Figure 3.1

Typical example of original oscilloscope records showing increased firing of the neuron in response to mustard oil and its inhibition by the NK_2 antagonist L-659,874.

The trace shows background spontaneous activity of this (multireceptive) neuron prior to testing. B shows activity 5 min after topical application of 7% mustard oil across the extent of the brush receptive field of the cell as determined by innocuous brush-evoked firing. In this example, mustard oil-evoked activity was sustained at this elevated level for a prolonged period without re-application. C shows activity 2 min after ionophoresis of L-659,874 was initiated, 18 min following the application of mustard oil. D shows recovery, 10 min after L-659,874 ionophoresis was terminated at the end of test C.

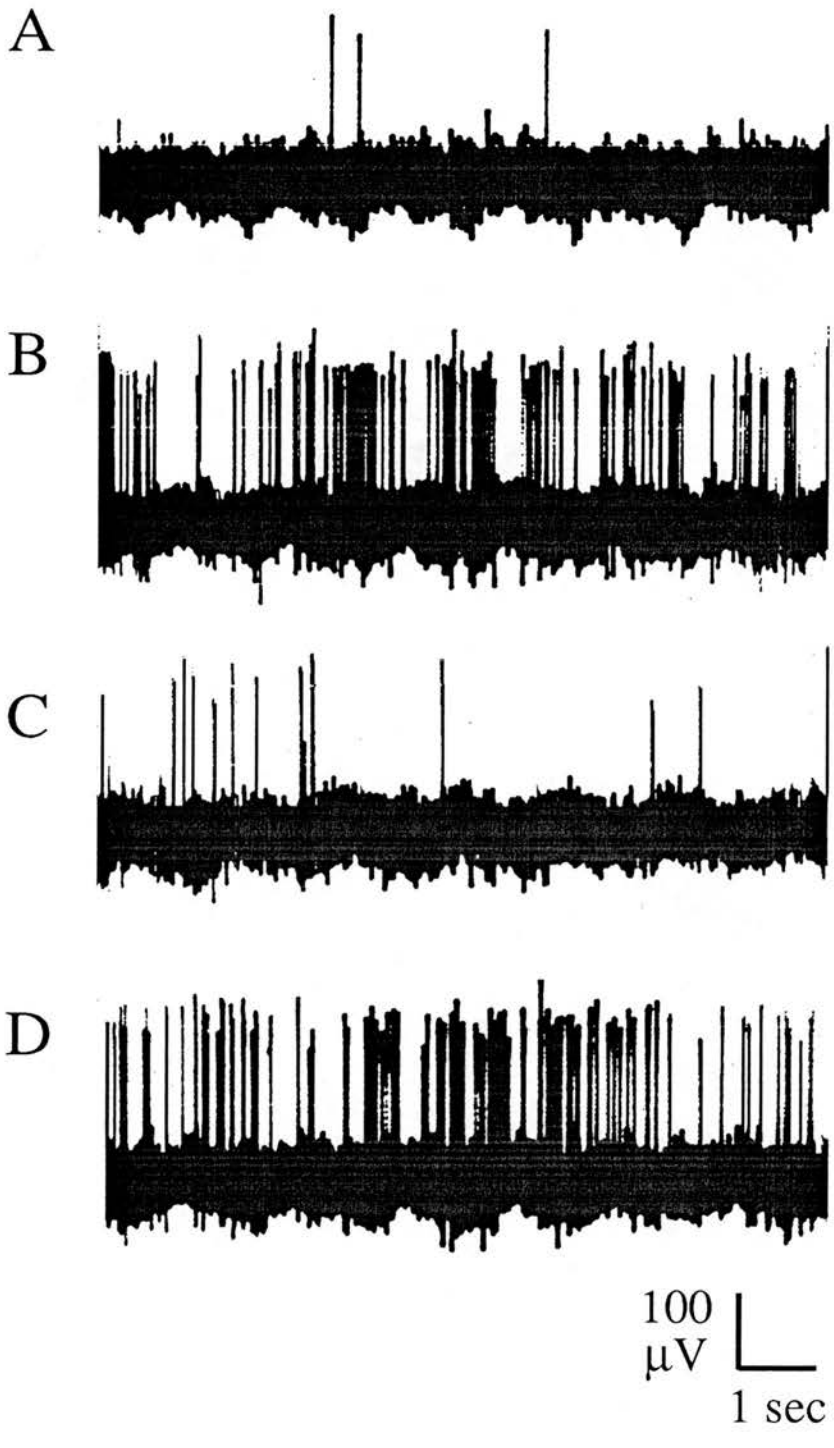

A
B
C
D
100
μV
1 sec

Figure 3.2

Typical inhibitory effects of the NK_2 antagonist L-659,874 on mustard oil-evoked activity of laminae IV/V dorsal horn neurons.

The records display firing frequency of the neuron (as spikes per sec) and its activation by either a single application (Fig. 1B), or 3 consecutive prior applications (Fig. 1A) of mustard oil. In the upper trace it can be observed that ionophoretic application of NaCl had no effect whereas L-659,874 promptly caused greater than 50% inhibition of evoked activity. After terminating ionophoresis of L-659,874 rapid recovery was observed and the cell readily responded to D, L-homocysteic acid (DLH) used as a presumed direct excitatory stimulus. In the lower trace it can be seen that mustard oil-evoked activity was progressively inhibited (and the inhibition maintained for longer periods) by ionophoresis of L-659,874 at increasing currents.

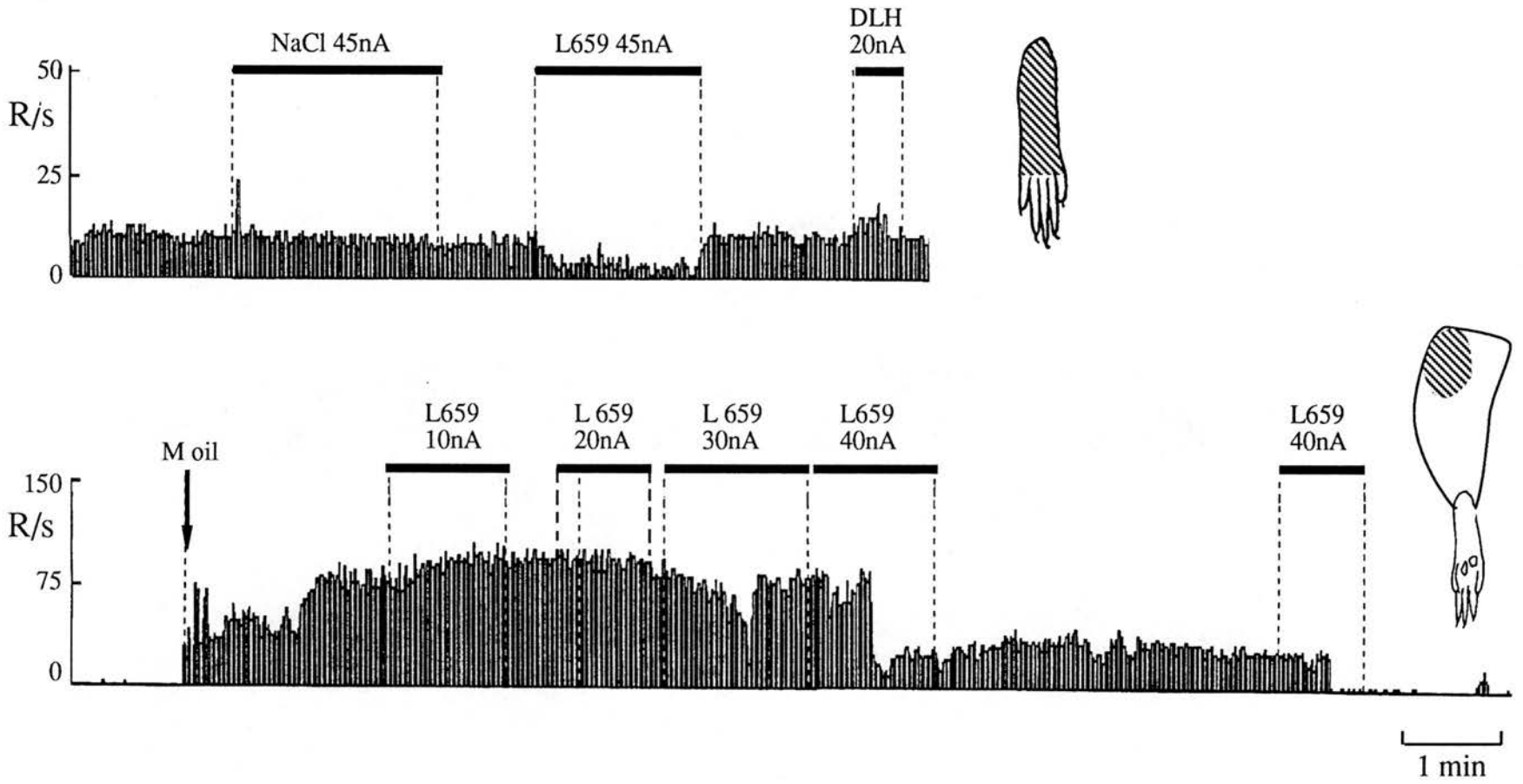

NaCl 45nA
L659 45nA
DLH
20nA
50
R/s
25
0
M oil
L659
10nA
L 659
20nA
L 659
30nA
L659
40nA
L659
40nA
150
R/s
75
0
1 min

Figure 3.3

Typical examples of the lack of effect of NK_1 antagonists, generally-observed on the mustard oil-evoked activity of laminae IV/V dorsal horn neurons.

The large increases in firing frequency elicited by mustard oil were unaffected by the NK_1 antagonists L-668,169 (A) and GR 82334 (B) applied after the mustard oil-induced activity had reached a steady state. In each case, the NK_2 antagonist L-659,874 caused inhibition. This effect was transient in example (A) and more long-lasting in (B), where recovery was seen after a delay of 3 min (not shown).

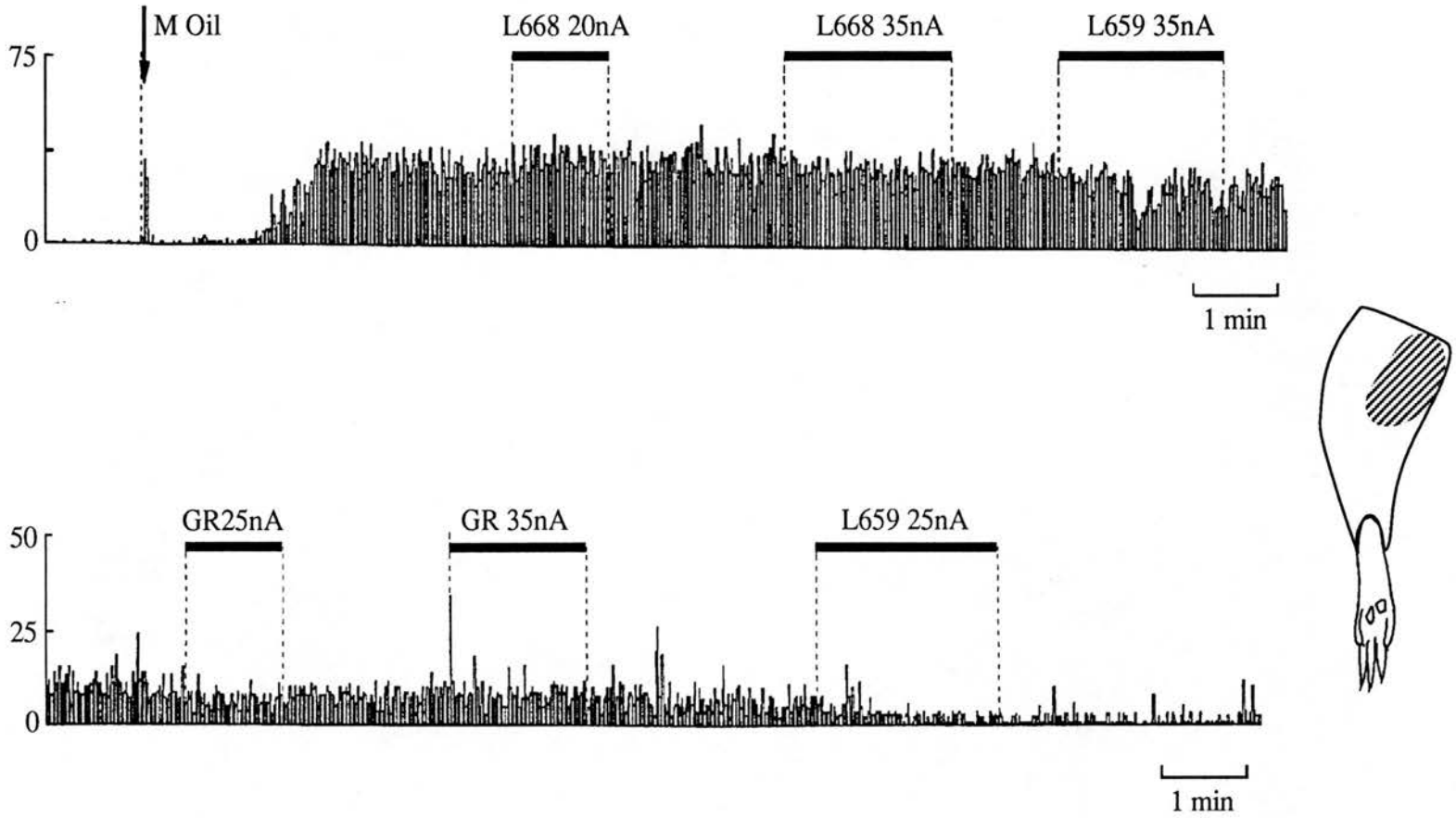

M Oil
L668 20nA
L668 35nA
L659 35nA
75
0
1 min
GR25nA
GR 35nA
L659 25nA
50
25
0
1 min

Figure 3.4

Typical example of a neuron responding to cutaneous application of mustard oil with a slow build up in firing frequency.

An example of a neuron which responded to cutaneous application of 5-20% mustard oil with a slow build up in firing frequency. The steady elevated firing rate was achieved by repeated application of mustard oil (3-5 times to the same site over a period of 10-15 minutes).

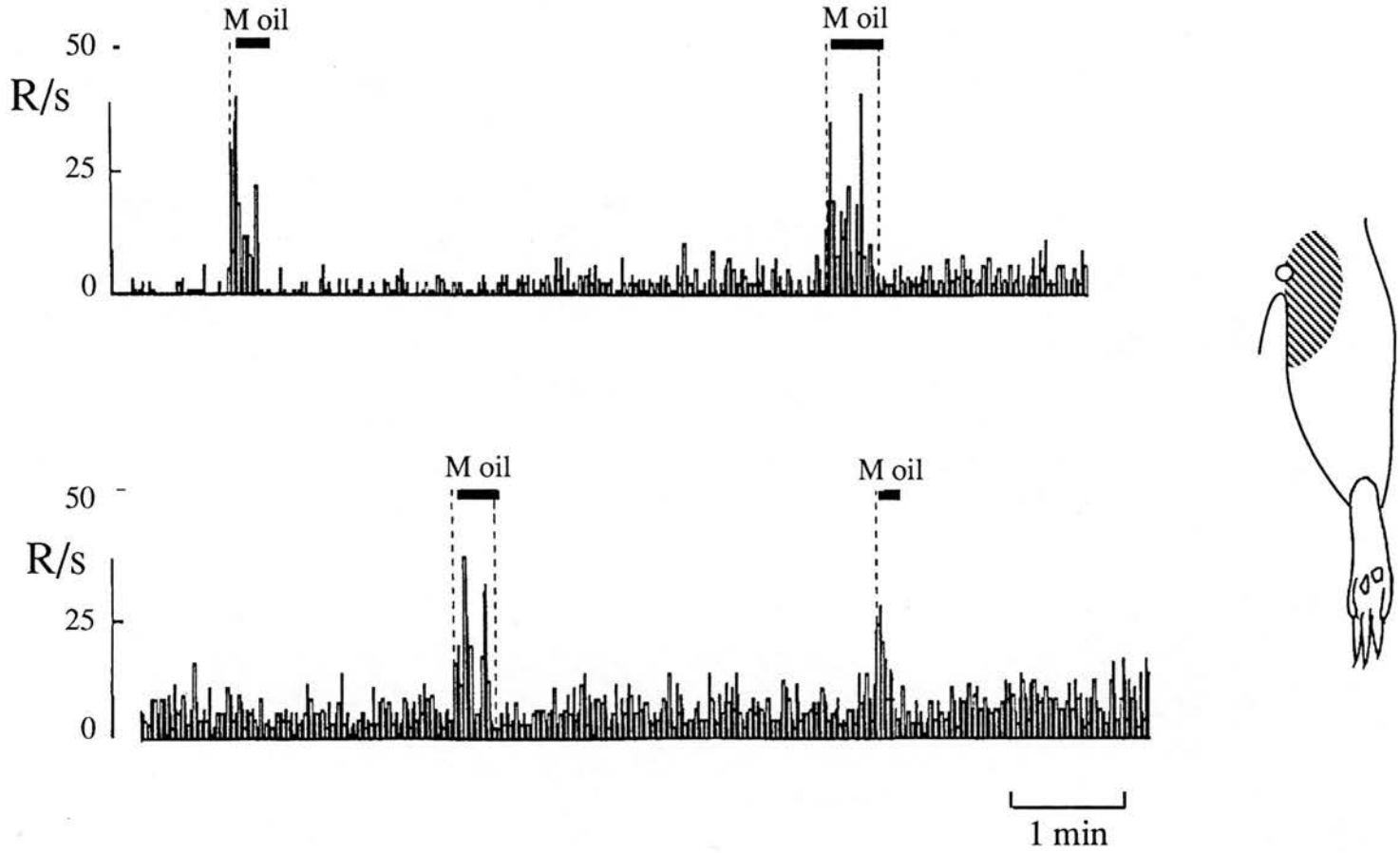
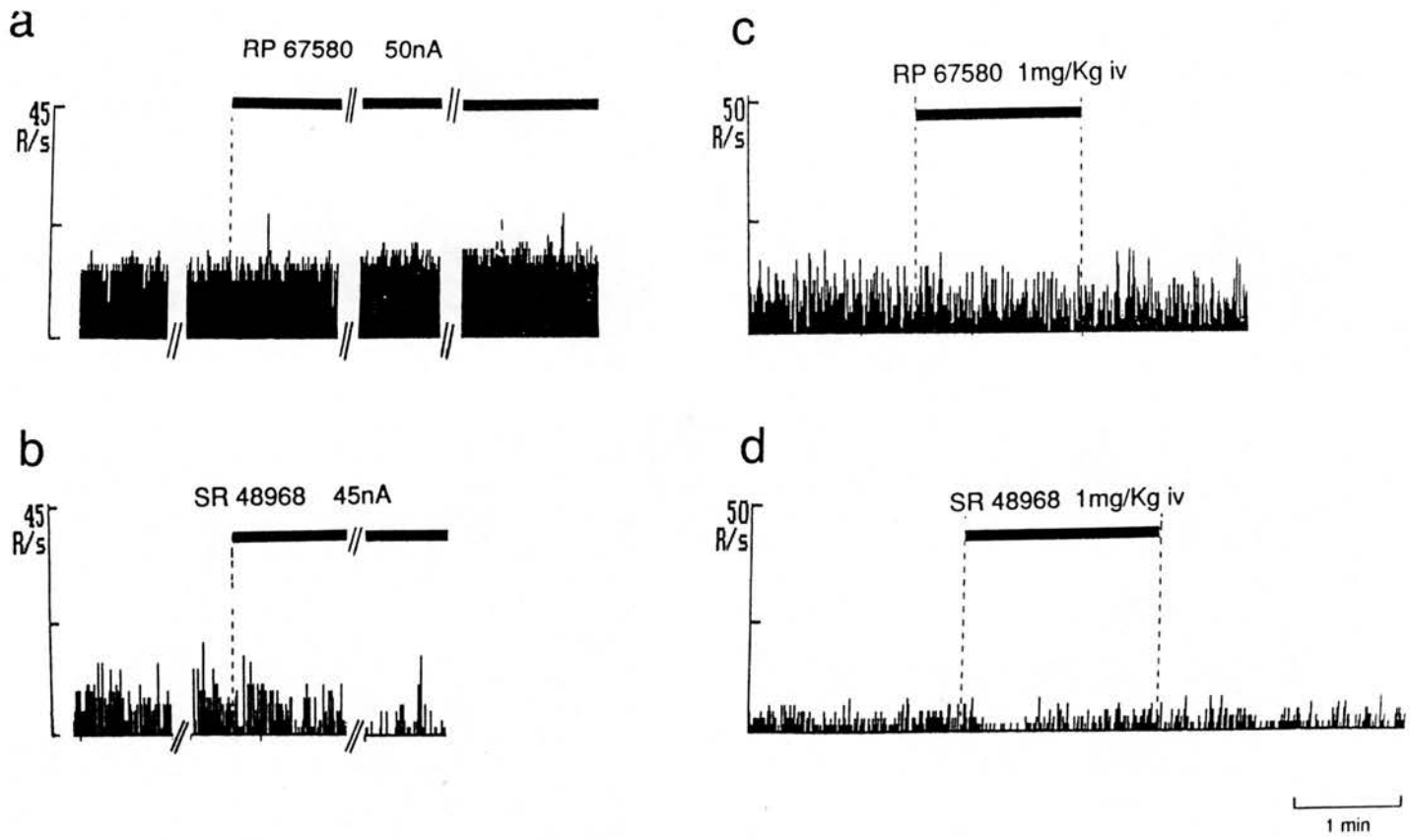

M oil
M oil
50
R/s
25
0
M oil
M oil
50
R/s
25
0
1 min

Figure 3.5

Typical effects of ionophoretic or intravenous administration of NK_1 and NK_2 non-peptide antagonist.

Effects of the NK_1 antagonist, RP 67580 and the NK_2 antagonist, SR 48968 on ionophoretic application in (a) and (b) respectively, or on intravenous administration in (c) and (d) respectively on the mustard oil-induced activity of multireceptive dorsal horn neurons.


a
RP 67580 50nA
45
R/s
b
SR 48968 45nA
45
R/s
c
RP 67580 1mg/Kg iv
50
R/s
d
SR 48968 1mg/Kg iv
50
R/s
1 min

cells there was a partial recovery to approximately 50 % of pre-drug controls.

Analysis was carried out off-line and the effect of SR 48968 on the mustard oil-evoked activity was calculated for each 1 minute ionophoresis period. The number of spikes integrated into each 1 minute ionophoresis period were expressed as a percentage of the pre-drug mustard oil-evoked activity (again calculated over 1 minute). No other drug was tested after intravenous injection of SR 48968.

3.4.6 Effect of NK_1 Antagonists on the Mustard Oil-Induced Activity.

The effect of NK_1 antagonists on the mustard oil-evoked activity was investigated using again two methods of administration.

(a) Effect of Ionophoretically-Applied NK_1 Antagonists

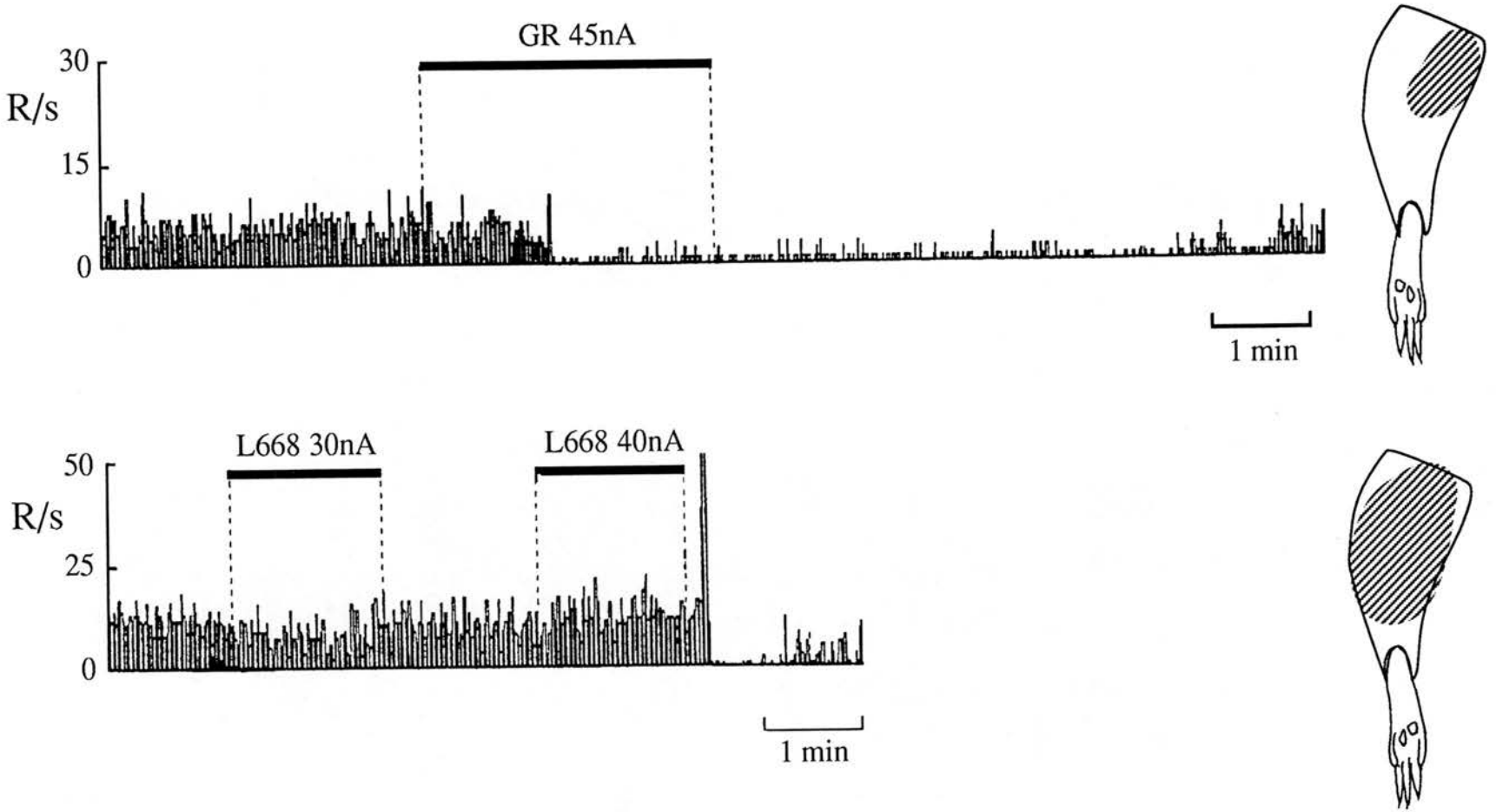
The effect of two selective NK_1 selective peptide antagonists L-668,169 (Regoli *et al,* 1987) and GR 82334 (Hagan *et al,* 1991) and one highly selective non-peptide antagonist RP 67580 were investigated (Garret *et al,* 1991) on mustard oil induced activity. L-668,169 and GR 82334 when ionophoresed at 20-80nA, rarely inhibited mustard oil-evoked activity (in 1 out of 11 and 3 out of 11 cases respectively). L-668,169 is reported to show 80-fold and 25-fold selectivity for NK_1 over NK_2 and NK_3 receptors respectively (McKnight *et al,* 1988) and equivalent values for GR 82334 are greater than 170-fold in each case (Hagan *et al,* 1991) Although L-668,169 is undoubtedly less rat selective than GR 82334 and RP 67580 (Beresford *et al,* 1992) it has been shown to have effects on the responses of dorsal horn neurons in rats (Fleetwood-Walker *et al,* 1991). In 17 cells tested with both NK_1 and NK_2 antagonist, there was only one example where both were clearly effective. The mustard oil-evoked activity in many cells (n=15) was unaffected by NK_1 antagonists, but inhibited by the NK_2 antagonist L-659,874 (Figure 3.3). In a small number of examples (n=4) there was unequivocal inhibition of activity by NK_1 antagonists (Figure 3.6), although in the one effective example with L-668,169, inhibition was not well related to the time of administration of the drug (Figure 3.6b). In this example the cell was lost while waiting for recovery.

In contrast to the two peptide antagonists investigated, the highly selective non-peptide antagonist RP 67580, which was ionopohoretically administered in an identical manner, in a current range of 30-85nA, gave entirely dissimilar results. In 3/4 cells examined, there was a 78.2 ± 11.3% decrease in the mustard oil-

Figure 3.6

Examples of the occasionally-observed inhibitory effect of NK_1 antagonists on mustard oil-evoked activity of laminae IV/V dorsal horn neurons.

A clear-cut inhibition of mustard oil-evoked firing was seen in 3 out of 11 cases with GR 82334, as in example (A). Delayed recovery over 30-60 minutes was observed. In only one example, (B), was there any evidence for inhibitory effects of L-668,169 and even there the inhibition was not closely matched to the time of drug application.


GR 45nA
30
R/s
15
0
1 min
L668 30nA
L668 40nA
50
R/s
25
0
1 min

evoked activity (mean ± s.e.m.) ($P<0.05$, Mann-Whitney U-test) from pre-drug controls with no observable recovery in any of the three neurons examined. The apparent discrepancy in results between peptide and non-peptide antagonists may be due to the small sample used in the study of the non-peptide antagonist.

Analysis was carried out 'off-line' and the effect of L-668,169, GR 82334 and RP 67580 on mustard oil-induced activity calculated as in section 3.3.2.

(b) Effect of Intravenous Application of NK_1 Antagonists

The non-peptide NK_1 antagonist RP 67580, when intravenously injected, gave entirely similar results to its ionophoretic administration. Intravenous administration was unfortunately examined in only two neurons, but both showed that a 1 mg/kg intravenous injection of the non-peptide antagonist resulted in a marked inhibition of mustard oil-facilitated background activity to 32.25% of the pre-drug control levels (see Figure 3.5). The maximum inhibition of mustard oil-evoked activity usually occurred 2-3 minutes after intravenous administration of the non-peptide drug, but recovery was not observed in either of the two cells examined

Analysis was carried out off-line and was similar to the methods described (section 3.3.2).

3.5 DISCUSSION

An *in vivo* experimental model of short-term sustained activation of C-fibre nociceptive afferents was employed using unilateral cutaneous application of the chemical algogen mustard oil. It provided a clear and reproducible model of behavioural nociception (see Cleland *et al*, 1994), with which to assess the effects of locally administered tachykinin receptor antagonists.

The neurons studied were all of a multireceptive nature, being responsive to both innocuous brush as well as noxious pinch and heat stimulation. There seemed to be a correlation between the ability of mustard oil to excite nociceptive neurons and the manner in which they respond to noxious heating of the skin. Those neurons which had a robust response to noxious cutaneous heat stimulation were used as these were more susceptible to showing a prolonged increase in firing upon cutaneous application of mustard oil. Their position was substantiated by both depth measurements and blue spot histology and correspond to laminae IV/V of the dorsal horn. Control studies demonstrated that the drug effects seen were not due to any potential adverse effects of vehicle (0.3%

dimethylformamide). The general excitant DLH also caused clear excitation, reducing the possibility that recordings were from primary afferent fibres (Goodchild *et al*, 1982) and NaCl current control experiments were unable to reproduce any of the drug-induced responses described below (see Figure 3.2).

The laminae IV/V neurons generally exhibited a low level of background activity (less than 1Hz) before application of mustard oil. This was an important consideration as higher levels would have masked any response to mustard oil. Following the repeated application of this chemical algogen (diluted to 8% in paraffin oil) onto the outlined receptive field area, the multireceptive neurons displayed a marked, facilitated increase in background firing activity resulting in a medium-term sustained nociceptive state. The background was consistently maintained at a steady level before the pharmacological testing was carried out. Some cells had an immediate increase in firing rate after one application of mustard oil which remained sustained for a prolonged period; however, in most cases, the steady elevated firing rate was achieved by repeated application of the algogen 3-5 times to the same site over a period of 10-15 minutes. The reason(s) for this differing sensitivity between neurons is unclear, perhaps neurons that are initially only slightly responsive are then sensitised to mustard oil (see Kress *et al*, 1992). However, this apparent different sensitivity to mustard oil had no obvious effects on the pharmacological results obtained.

In contrast, when mustard oil, at dilutions of up to 20%, was applied to the receptive fields on glabrous skin there was a minimal effect on the firing rates of multireceptive neurons. This lack of effect of mustard oil on glabrous skin has been attributed to a failure of mustard oil to penetrate the skin adequately (Harris and Ryall, 1988) and consequently, only neurons with receptive fields on the thinner hairy skin were used in these studies. Infrequently, there was no increase in firing rates after cutaneous mustard oil application to hairy skin, even in cells with nociceptive heat responses, suggestive that there may be sub-populations of multireceptive C fibres that are unresponsive to this chemical algogen or alternatively lack of access to the appropriate sites. Importantly, on no occasion was a decrease in firing rate seen following the topical application of mustard oil. In the present experiments, despite careful mapping of the receptive field for each neuron, there was no clear evidence for receptive field expansion following peripheral application of mustard oil, as described by Woolf and King, (1990); Woolf and Thompson, (1991). This perhaps be explained by the slight differences between experimental designs. In contrast to the present experiments, Woolf and King, (1990) applied mustard oil to regions close to but outwith the overt firing

zones or alternatively, the mustard oil activity was studied over prolonged periods of up to 90 minutes (Woolf and Thompson, 1991).

The results presented here show that NK_2 but not NK_1 receptors, play a crucial role in mediating the sustained excitation of laminae IV/V neurons elicited by cutaneous application of mustard oil. The evidence suggests that this is specifically an NK_2 receptor-mediated event as the selective peptide and non-peptide NK_2 antagonists, L-659,874 and SR 48968 respectively show extremely good selectivity for NK_2 over NK_1 and NK_3 receptors (McKnight *et al*, 1988; Williams *et al*, 1988; Advenier *et al*, 1992; Emonds-Alt *et al*, 1992,1993). However the possibility of unidentified side effects playing a role cannot be ruled out.

A stable and continuous mustard oil-induced increase in background activity was attained before ionophoresis of antagonists was initiated. Ionophoresis of the highly selective NK_2 antagonist L-659,874 resulted in a marked and relatively maintained inhibition in the mustard oil-induced activity, which began after a variable onset delay, sometimes immediate but more regularly taking a period of up to two minutes before maximum inhibition was achieved. The difference between neurons is possibly due to inconsistency in the distances required for the antagonist to reach its site of action. As L-659,874 is a peptide antagonist, it may be degraded, thus these results were further substantiated using both ionophoretic and intravenous administration of the highly potent non-peptide antagonist SR 48968 (Emonds-Alt *et al*, 1992). Both regimes yielded results entirely similar to those achieved with L-659,874, with an identical pattern of sustained yet recoverable inhibition of the mustard-oil induced firing rate, the only difference being the longer recovery time required after intravenous administration.

Conversely, there were mixed results (both excitatory and inhibitory) following administration of NK_1 receptor antagonists. Ionophoretic administration of each of the two selective peptide NK_1 receptor antagonists, L-668,169 and GR 82334, rarely inhibited the mustard oil-induced activity. GR 82334 does show higher affinity for the rat NK_1 receptor site (Beresford *et al,* 1992) than L-668,169 (McKnight *et al,* 1988). However these observations show that ionophoresis of both antagonists lead to a similar outcome suggesting that NK_1 receptors generally do not play a role in the acute facilitatory response to mustard oil. Nevertheless, in a small number of examples there was unmistakable inhibition of activity by NK_1 antagonists. Indeed, although this was relatively rare with L-668,169, which shows low potency at the rat NK_1 receptor (Patacchini *et*

al, 1992), GR 82334, which shows high selectivity for the rat NK_1 over NK_2 and NK_3 receptors (Hagan *et al,* 1991), was more effective in yielding an inhibition following its ionophoretic administration. The reasons for this discrepancy are unclear, however it may be that NK_1 receptors are of greater importance in mediating responses to noxious stimuli in more prolonged, and inflammatory pain states which have been unwittingly attained in this small sample of neurons. Moreover, in accord with these results are those attained with both ionophoretic and intravenous administration of the potent non-peptide NK_1 receptor antagonist, RP 67580 (Garret *et al,* 1991). Both administration techniques resulted in a significant decrease in the mustard oil-evoked activity with no observable recovery. Only a small population of neurons was sampled which may be the reason for the apparent discrepancy between these results and the majority obtained using peptide NK_1 antagonists.

It is of course also possible that the localised route of administration of drugs here did not allow the NK_1 antagonists to access their (perhaps slightly more distant) sites of action. SP is a potent excitant of laminae IV/V nociceptive neurons (Henry, 1976) and on intrathecal administration SP produces behaviour suggestive of noxious irritation (Hylden and Wilcox, 1981). In addition, intrathecal injection of certain peptide and non-peptide NK_1 antagonists elevate behavioural response thresholds (Cridland and Henry, 1986, 1988; Garrett *et al*, 1991; Murray *et al*, 1991; Yamamoto and Yaksh, 1991; Yashpal *et al*, 1993). However, other studies find no role for SP as a primary transmitter of nociceptive information (Doi and Jurna, 1981; Piercey *et al*, 1981; Gamse and Saria, 1986. These and further observations that pinch rather than noxious heat stimuli release SP into dorsal horn (Duggan *et al*, 1988; Kuraishi *et al*, 1989) and that SP-like immunoreactivity is localised in a proportion of small diameter primary sensory units which have more recently been identified as A fibre high threshold mechanoreceptor units or glabrous skin C-mechanoheat units (Lawson *et al*, 1994). Surprisingly, in contrast to widely held assumptions, SP-LI was not detectable in C polymodal nociceptor units located in hairy skin (Lawson *et al*, 1994). This is consistent with the possibility that a somewhat different subset of nociceptive afferents is involved in pinch-evoked SP release from those in the heat- and mustard oil-evoked neuronal activity we have observed to be sensitive to NK_2 antagonists.

Nevertheless, neither brief neuronal responses to pinch (Fleetwood-Walker *et al*, 1990, 1991, 1992); nociceptive flexor reflexes (Wiesenfeld-Hallin *et al*, 1990), nor capsaicin-induced responses in an *in vitro* spinal cord-dorsal root

ganglion preparation (Nagy *et al*, 1993) appear to be directly prevented by NK_1 antagonists. Instead the facilitation of nociceptive flexor reflex elicited by conditioning C-fibre stimulation of the sural nerve is inhibited by the antagonist spantide II which shows a modest selectivity for NK_1 over NK_2 receptors (Maggi *et al*, 1991). This is consistent with evidence that SP (NK_1) antagonists prevent the secondary behavioural hyperalgesia following heat injury (Coderre and Melzack, 1991) and the late prolonged but not the brief epsps induced in dorsal horn neurons by C-fibre activation or noxious stimuli (Urban and Randic, 1984; De Koninck and Henry, 1991).

In view of evidence for differences in the absolute potency of certain NK_1 antagonists between species, we tested GR 82334, which in contrast to L-668,169 (and indeed also spantide II) shows only slightly reduced potency in the rat bladder compared to guinea-pig and rabbit tissue bioassays (Beresford *et al*, 1992; Patacchini *et al*, 1992). Nevertheless, the present results with a medium-term prolonged chemical algogenic stimulus support previous observations on responses to brief noxious thermal stimuli (Fleetwood-Walker *et al*, 1990, 1991, 1992) in their marked sensitivity to NK_2 rather than NK_1 receptor agents; thereby suggesting that somewhat different inputs or circumstances are involved here from those in experiments showing NK_1 sensitivity. Consistent with this theme, the facilitation of a nociceptive reflex induced by intrathecal administration of NKA or by conditioning stimulation of the gastrocnemius nerves at C-fibre intensity (Xu *et al*, 1991) and the excitation of dorsal horn neurons evoked by application of capsaicin to the dorsal root ganglia (Urban *et al*, 1992) are all inhibited by selective NK_2 receptor antagonists. The detailed characteristics and precise location of this NK_2-like receptor in dorsal horn remain to be ascertained.

The results presented here show that the NK_2, but not the NK_1 receptors, play a crucial role in mediating the sustained excitation of laminae IV/V neurons elicited by cutaneous application of mustard oil; a medium-term prolonged activation of nociceptive C and perhaps Aδ afferents (Woolf *et al*, 1986; Heapy *et al*, 1987; Harris and Ryall, 1988).

CHAPTER 4:
Electrophysiological Examination of Evoked Sensory Responses in Laminae III-V Rat Dorsal Horn Neurons Before and After Application of Mustard Oil.

4.1 AIMS

The aim of these experiments was, (using the technique of extracellular recordings from laminae III-V multireceptive dorsal horn neurons), to examine the effects of local ionophoresis of NK_1 and NK_2 antagonists on sensory responses (both noxious and innocuous) before and after prolonged cutaneous application of mustard oil, which induces central sensitisation and hyperalgesia.

4.2 MATERIALS

Animals: Male Wistar rats were obtained from Charles River UK Ltd, Margate, Kent, UK.

Anaesthetics: Alpha-chloralose and urethane were obtained from Sigma Chemical Company, Poole, Dorset, UK; Halothane (flurothane) was obtained from ICI Pharmaceuticals, Macclesfield, Cheshire, UK.

Laboratory Chemicals: Standard laboratory chemicals were of Analar grade and from the following suppliers: Sodium chloride (NaCl), DLH (D, L, homocysteic acid) and Pontamine Sky Blue (PSB) were obtained from Sigma Chemical Company, Poole, Dorset, UK; allyl-isothiocyanate (mustard oil) was purchased from Aldrich Chemical Company Ltd, Gillingham, Dorset, UK; Halothane (Flurothane) was obtained from ICI Pharmaceuticals, Macclesfield, Cheshire, UK. Agar was obtained from Oxoid Ltd, Basingstoke, Hampshire, UK and paraffin was purchased from Scotlab, Lanarkshire, UK.

Drugs: The tachykinin antagonists, acetyl-Leu,Met,Gln,Trp,Phe-NH_2 (L-659,874) and cyclo(Gln,D-Trp,Me-Phe,(R)Gly[ANC-2]Leu,Met)$_2$, (L-668,169) were obtained from Cambridge Research Biochemicals & [D-Pro^9[spiro-γ-lactam]-Leu^{10},Trp^{11}]physalaemin-(1-11), (GR 82334) was a gift from Glaxo Group Research; 2-[1-imino-2-(2-methoxyphenyl)ethyl]-7,7 diphenyl-4 perhydroisoindolone (RP 67580) was a gift from Dr C Garret, Rhone-Poulenc Recherché-Development, France and (S)-N-methyl-N[4-(4-acetylamino-4-phenyl

piperidino)-2-(3,4-dichloro phenyl)butyl] benzamide (SR 48968) was a gift from Dr X Emonds-Alt, Sanofi Recherché, France.

4.3 METHODS

4.3.1 General Methods

See Chapter 2 for details of animals, surgical techniques histological determination of laminar location and general procedures for recording & ionophoresis. Extracellular recordings from single laminae III-V dorsal horn neurons were made through the central barrel of a 7-barrelled microelectrode containing 4M NaCl, pH 4.0-4.5 and drugs were ionophoresed from the side barrels. One side barrel contained 1M NaCl (pH 4.5-5.0), for automatic current balancing, another barrel was filled with PSB for marking the position of recording sites and the other electrode barrels contained various combinations of the following selective NK_1 and NK_2 antagonists:- L-659,874 and L-668,196 (0.1mM in 0.3% DMF, pH 4.0-4.5); GR 82334 (1mM in distilled H_20, pH 4.0-4.5); RP 67580 and SR 48968 (1mM in distilled H_20, pH 4.0-4.5).

4.3.2 Quantification of Sensory Responses Before and After Application of Mustard Oil.

Neurons under examination all had multireceptive cutaneous receptive fields on the ipsilateral hindlimb. Neurons were initially detected by widespread manual brushing of the ipsilateral hindlimb whilst moving the recording electrode with the microdrive up and down the track under examination. Once an excitatory response to manual innocuous brush was detected the often more restricted noxious heat receptive field was localised. Neurons were used only when equally matched responses were attained for both innocuous brush and noxious heat.

All of the multireceptive neurons used had excitatory receptive fields on the ipsilateral hindlimb, paws and toes and the controlled noxious and innocuous stimuli used were applied to adjacent sites within the ipsilateral cutaneous field (see Figure 2.1). The innocuous cutaneous stimulus used was a manually operated, soft, paintbrush which was rotated for a 10 - 15 second period. The noxious thermal stimulus used was a thermocouple-controlled radiant heat lamp, precisely raising skin temperature from 30°C - 48°C (the exact maximum temperature could be set for different neurons) over a 3 second rise time and maintained at the maximum temperature for a further 5 - 10 seconds. A lesser

duration was used for noxious heat than innocuous brush to avoid any sensitisation.

As testing was to be carried out for a considerable time it was important that the limb under study was held in exactly the same position. This was achieved by fixing the position of the limb with a clamp which ensured that the cutaneous stimuli remained in the same position for the duration of the test. The brush and heat stimuli were then cycled over 3 minute time periods to allow full recovery of the neuron between tests and to avoid any unnecessary sensitisation of the responses.

Throughout the tests, care was taken to ensure that the recorded spike height remained significantly greater than the background noise, occasionally this meant moving the position of the recording electrode by a few μm in order to get closer to the neuron during the testing procedure.

First, the control responses to brush and heat were carried out. At least two well-matched sets of brush and heat responses were achieved before any further testing. The testing was abandoned at this stage if well-matched brush and heat responses could not be found. The drug under study was ionophoresed for 1 minute before the start of each test cycle and continued throughout the cycle, starting at a low ejection current and increasing by 10 or 20 nA stepwise each cycle. After the drug application, full recovery of responses was achieved (from 5 to 30 minutes) before the next drug under study was tested.

Once all the drugs had been tested in this manner, the cell was left for 30 minutes to fully recover before application of the chemical algogen mustard oil. An 8% solution of mustard oil (in paraffin oil) was painted onto the receptive field area of the hindlimb and foot using a soft paintbrush every 5 minutes until the activity had sufficiently increased as described in section 3.3. The control sensory responses to brush and heat were then completed in duplicate or triplicate until 2 matched sets of sensory responses were achieved in a similar fashion to that previously described. The drug under study was then ionophoresed for 1 minute before the sensory responses were tested once again and then this process was repeated, increasing the ionophoretic current in a stepwise manner by 10nA each cycle. After each drug application full recovery of responses was achieved before the next drug was tested.

4.3.3 Analysis

The neuronal firing was continuously monitored on FM tape (Racal) and firing rates were plotted on-line by computer (IBM PS/2-70-121) together with

stimulator and ionophoresis markers. The data was stored on the hard disk of the computer and analysed off-line by integrating the number of stimulus-induced action potentials in selected constant time intervals. Responses to mechanical brush were integrated over 10 second stimulus times, whereas responses to noxious thermal stimuli were integrated over a 10-15 second interval. The spontaneous activity prior to the response was always taken into account and, once removed from the control brush and heat values, these served as the 'normalised' controls and were expressed as a value of 100%. After drug treatment, again the brush and heat responses were normalised and expressed as a percentage of the control value.

After peripheral application of mustard oil to the receptive field, sensory responses to brush and heat were monitored firstly to elucidate the effect of the peripheral mustard oil itself and secondly to determine the drug effect on the mustard oil sensitised brush and heat responses. After application of the algogen the spontaneous activity was much higher than before but similarly, the background activity was subtracted from the responses to brush and heat to normalise the results. The drug effect on each of the mustard oil-sensitised brush and heat was expressed as a percentage of the control responses before mustard oil application. These can be seen in Figures 4.1, 4.2 and 4.3.

4.4 RESULTS

4.4.1 Characteristics and Receptive Field Properties of Neurons Tested

The present results were obtained from 18 neurons located in laminae III-V of the dorsal horn of 18 rats as identified by histological determination with PSB spots on transverse sections of the spinal cord. Laminar location was estimated as the depth recording on the microdrive which for laminae III-V was 250-600μm. Similar to Chapter 3, recording from neurons deeper than 600μm was rarely pursued as they normally resulted in an unsuccessful response to mustard oil.

All neurons recorded were multireceptive (responded to both innocuous brush and noxious heat and pinch). It was particularly important that the magnitude of responses to both noxious and innocuous stimuli were well matched where possible, in order to avoid any potential artefacts due to a differential sensitivity of submaximal and near-maximal responses to drugs. Thus only

responses which met this criterion were used. Additionally, a strong response to noxious heat appeared to be a prerequisite for a good response to mustard oil.

The cutaneous receptive fields of the mustard oil-activated neurons were always located on the foot, toes or flank of the ipsilateral hindlimb. It was important that the receptive field was located on hairy and not the glabrous skin as no 'sensitisation' of the neurons followed application of mustard oil to this, presumably as a result of poorer penetration of the latter region by the mustard oil. Receptive field size varied greatly between neurons, although this had no apparent influence on the responsiveness of the neuron to cutaneous application of mustard oil.

4.4.2 Control Responses To Sensory Stimuli

Two sets of stable control responses were achieved for both brush and heat. It was an essential requirement that the activity remained stable and that, where possible, responses to brush and heat were well matched in size.

4.4.3 Effects of Ionophoretically-Applied Neurokinin Antagonists on Sensory Responses Before and After Application of Mustard Oil.

Sensory responses to innocuous brush and noxious heat were compared both before and after the peripheral application of mustard oil. The effects of highly selective NK_1 antagonists GR 82334 and RP 67580 (Garret *et al,* 1991) and NK_2 antagonists L-659,874 and SR 48968 (Emonds-Alt *et al,* 1992) were also tested on the responses of these multireceptive neurons both before and after mustard oil.

(a) Pre-Mustard Oil Responses

The pre-mustard oil sensory responses were assessed in a similar protocol design to those described in Chapter 2. Briefly, once the receptive field had been found by widespread manual brushing, the sensory stimulators were set up in such a way that they would not move from the original position throughout the duration of the experiment. Once two sets of control responses had been attained, the drug under investigation was ionophoresed. The drug was ejected at a low current initially for 1 minute before the sensory responses were repeated, this cycling of responses was repeated every 3 minutes, each time increasing the ejection current by 10 or 20 nA until an effect of the drug was seen.

Records such as those shown in Figures 4.1, 4.2 and 4.3 were analysed off-line by integrating the number of stimulus-induced action potentials in selected

time intervals, which were kept constant for a particular response taking the spontaneous activity value from the spontaneous values. Results were expressed as a percentage of the pre-drug control value, which itself was expressed as 100%, for innocuous brush the responses were analysed over a 10 second period and the noxious heat was analysed over a 20-30 second period taking into account any long train of after-discharge. Statistical significance of the changes was assessed by the Wilcoxon Test.

The Effect of NK_1 Antagonists on Pre-Mustard Oil Responses.

The results were entirely consistent with previous studies using NK_1 antagonists (see Chapter 2). The responses to spontaneous activity and responses to noxious heat were not significantly different from control values ($P>0.05$) for either of the selective NK_1 antagonists. The vehicle (0.3% DMF in water) had no effect in 3 out of 3 neurons when ejected at up to 80nA for 12 minutes. However, in 6/7 and 6/8 neurons for GR 82334 and RP 67580 respectively, ionophoresis of these selective NK_1 antagonists resulted in a small but reliable enhancement of activity evoked by innocuous brush. Ionophoresis of GR 82334 at 30-80nA resulted in a significant enhancement of activity evoked by innocuous brush by $+54 \pm 7\%$ ($n = 9$) from the control value (see Figures 4.1, 4.3a and 4.4); in the remaining cell there were no detectable changes. Similarly, ionophoresis of RP 67580 at 30-85nA caused a small, but reliable enhancement of innocuous brush activity to 136 ± 8 ($n = 5$) of the control value (see Figures 4.2, 4.3b and 4.4) and in the remaining 2 cells there was no detectable change in activity due to ionophoresis of the antagonist.

The Effect of NK_2 Antagonists on Pre-Mustard Oil Responses

Before application of mustard oil to the receptive field area, both the NK_2 antagonists under study (L-659,874 and SR 48968) gave similar results (see Figures 4.3c,d and 4.4). Neither antagonist produced any significant change in the response to innocuous brush. Ionophoresis of L-659,874, over a current range of 15-55nA resulted in a brush response of $104 \pm 5\%$ mean $\pm$ s.e.m. ($n = 8$) compared to control. Similarly, over a current range of 40-65nA, ionophoresis of the non-peptide NK_2 antagonist SR 48968 resulted in an insignificant change in innocuous brush at $98 \pm 8\%$ mean $\pm$ s.e.m ($n = 5$) compared to pre-drug control. Neither drug produced any significant change in the background (spontaneous) activity. However, as previously described (Chapter 2), both the NK_2 drugs under

Figure 4.1

The effects of GR 82334 on sensory responses of corresponding laminae IV/V neurons before and after mustard oil application

A typical examples of the effects of the NK_1 tachykinin antagonist, GR 82334 on (a) brief sensory responses of laminae IV/V neurons, and (b) sensory responses of laminae IV/V neurons following sensitisation of neuronal responsiveness as a consequence of cutaneous application of mustard oil.

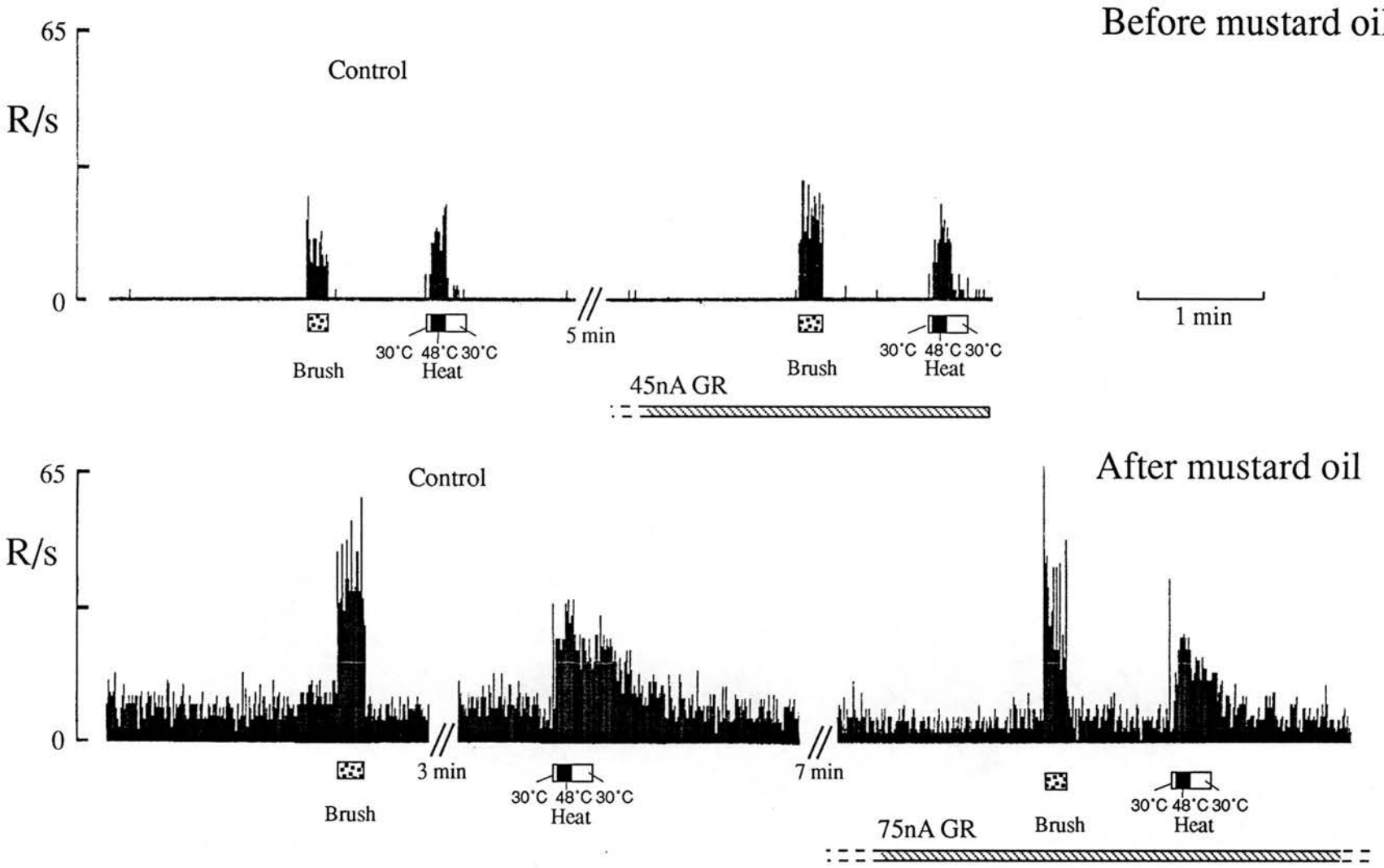

Before mustard oil
65
R/s
0
Control
Brush
30°C 48°C 30°C
Heat
5 min
Brush
30°C 48°C 30°C
Heat
1 min
45nA GR
After mustard oil
65
R/s
0
Control
Brush
3 min
30°C 48°C 30°C
Heat
7 min
Brush
30°C 48°C 30°C
Heat
75nA GR

Figure 4.2

The effects of RP 67580 on sensory responses of a laminae IV/V neuron before and after mustard oil application

The complete recording sequence from a laminae IV/V neurons demonstrating the effect of the NK_1 antagonist RP 67580 on (a) brief sensory responses of this neuron, and (b) sensory responses following sensitisation of neuronal responsiveness as a consequence of cutaneous application of mustard oil.

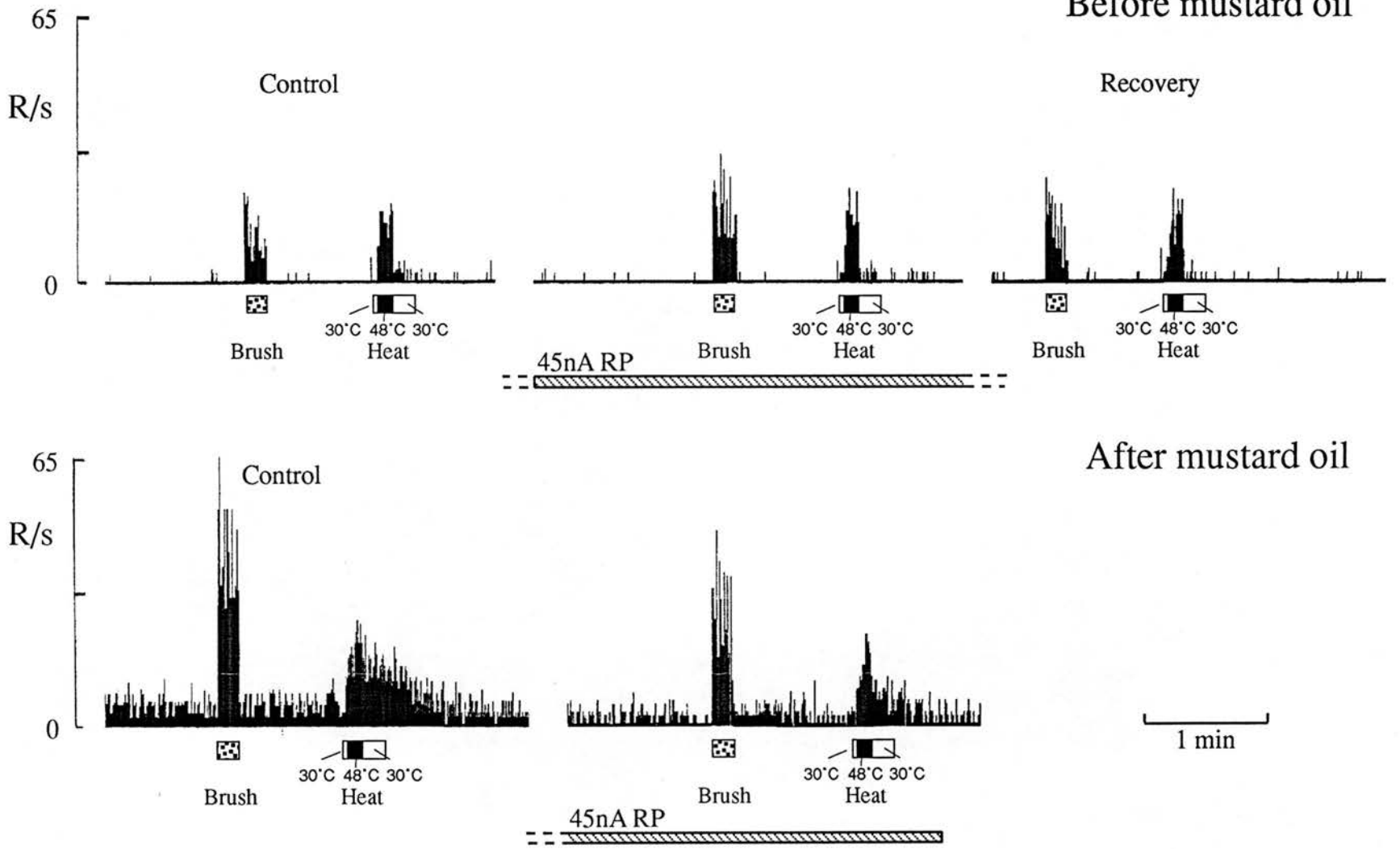

Before mustard oil
65
R/s
0
Control
Recovery
30°C 48°C 30°C
Brush
Heat
45nA RP
After mustard oil
1 min

Figure 4.3

The effects of tachykinin antagonists on sensory responses of laminae IV/V neurons before and after mustard oil application, expressed as values normalised to corresponding controls.

Mustard oil caused mean increases in responses of 212 ± 27% (n=17) for innocuous brush and 219 ± 27% (n=18) for noxious heat (to 48°C). The drugs administered ionophoretically were as follows :

(a) GR 82334: 30-80nA prior to mustard oil (n=6-7), 25-85nA following mustard oil (n=5-6).

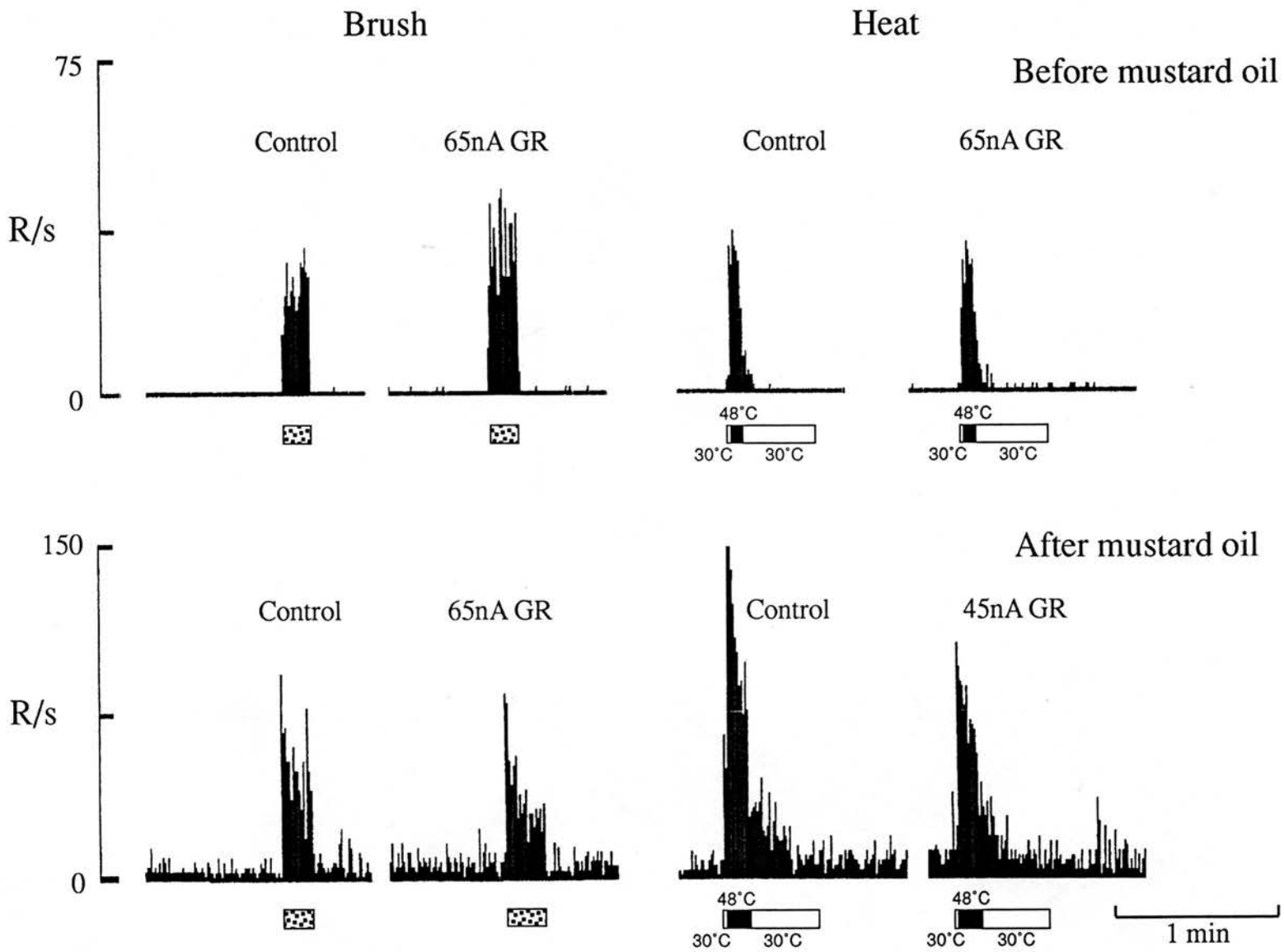
(b) RP 67580: 30-85nA prior to mustard oil (n=4-7), 40-85nA following mustard oil (n=4).

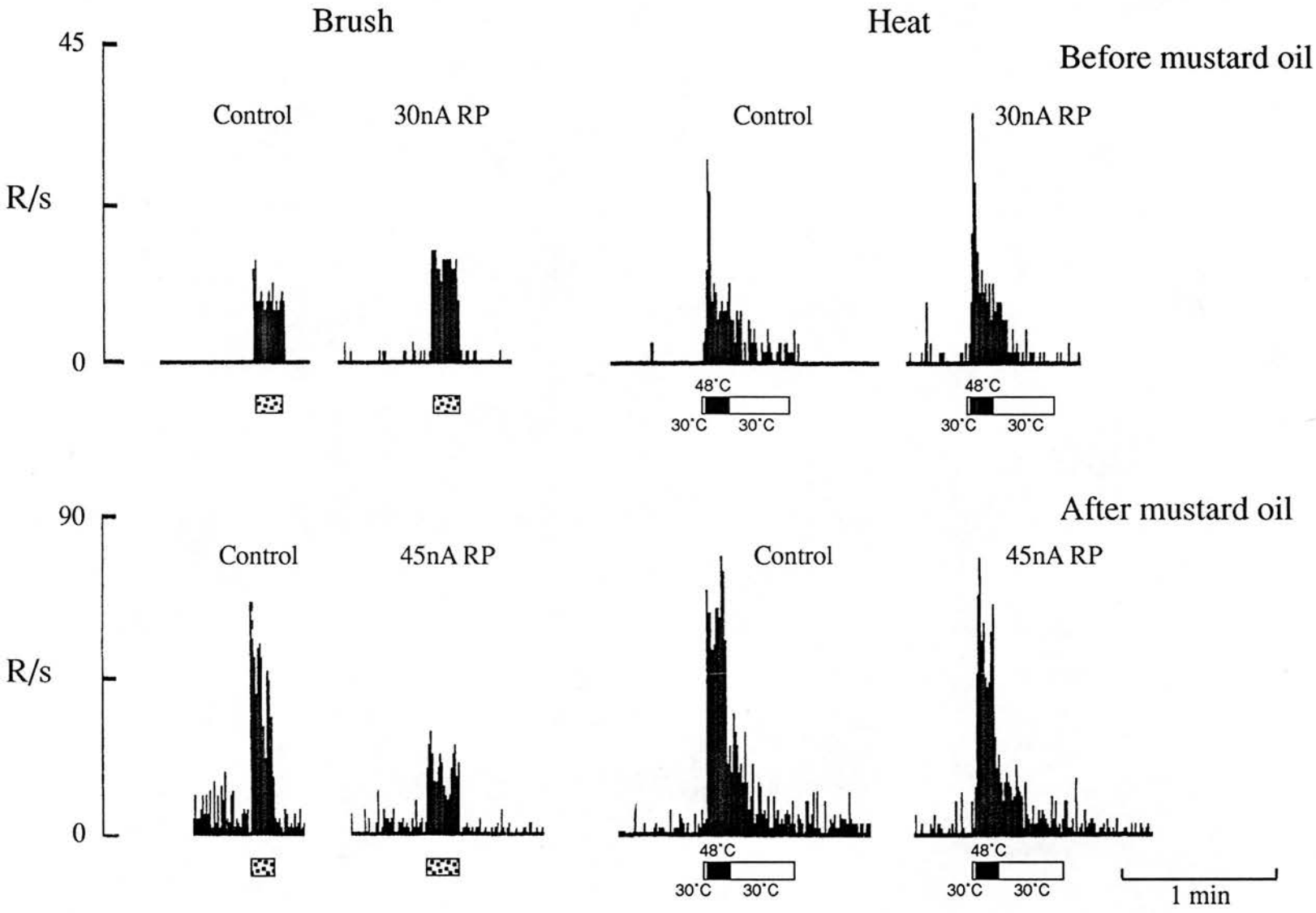
(c) L-659,874: 15-60nA prior to mustard oil (n=5-8), 15-95nA following mustard oil (n=3-5).

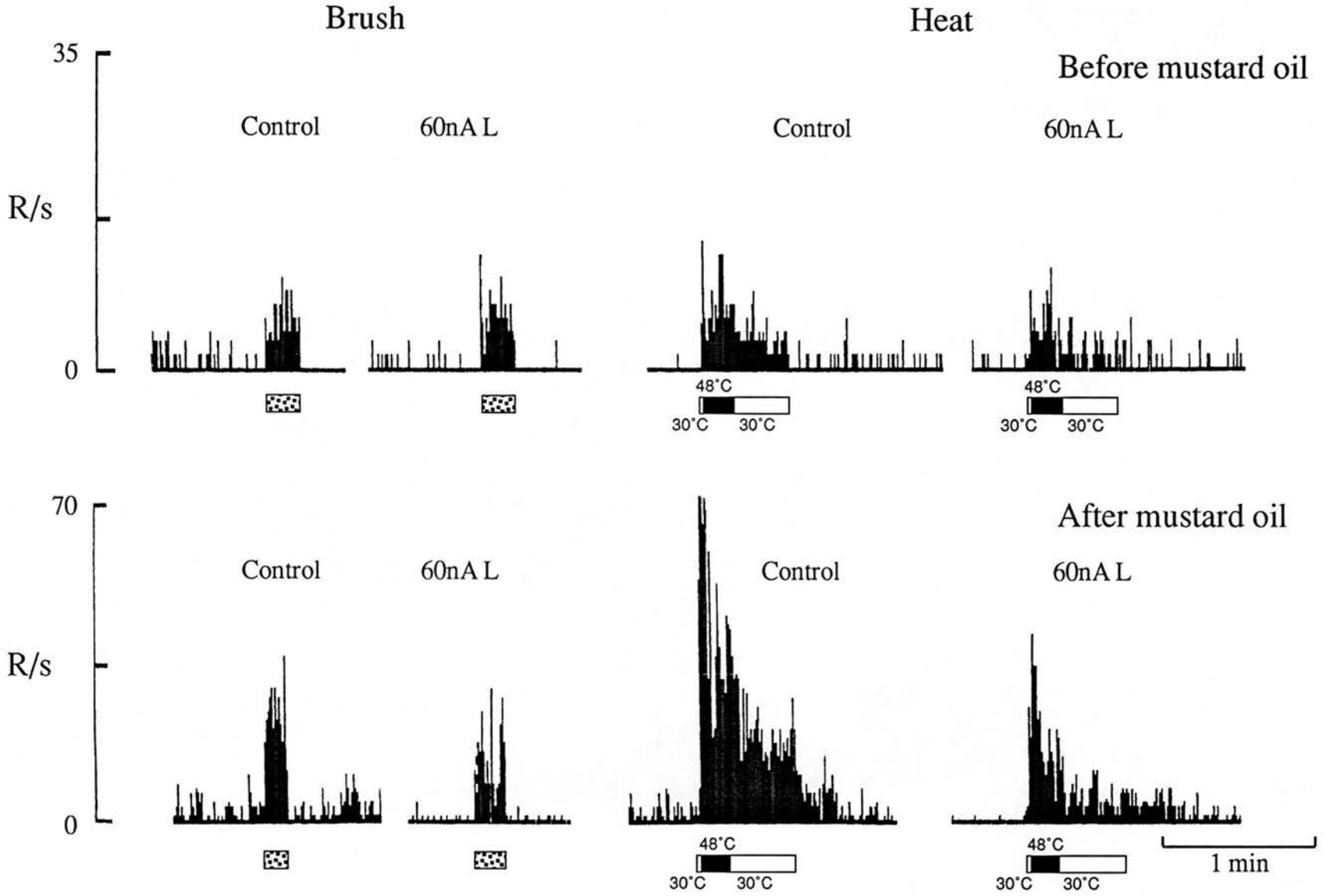
(d) SR 48968: 40-65nA prior to mustard oil (n=4-5), 40-70nA following mustard oil (n=4).

There was no consistent difference in the currents required for the effects described prior to mustard oil. The statistical significance of drug effects compared to their concurrent controls was assessed by the Wilcoxon Signed Ranks test (* $P \leq 0.05$). The statistical significance of differences in drug effects prior to, or following mustard oil was assessed by Mann-Whitney U-test († $P < 0.05$).

Due to the practical difficulties ensuing from the complex and prolonged protocol, it was possible only in a limited number of cases to obtain complete recording sequences of any given neuron, both before and after mustard oil. Each result was however, entirely typical of those obtained in any particular case (n=3-8).


Brush
Heat
Before mustard oil
75
R/s
0
Control
65nA GR
Control
65nA GR
48°C
30°C
30°C
48°C
30°C
30°C
After mustard oil
150
R/s
0
Control
65nA GR
Control
45nA GR
48°C
30°C
30°C
48°C
30°C
30°C
1 min


Brush
Heat
Before mustard oil
45
R/s
0
Control
30nA RP
Control
30nA RP
48°C
30°C
30°C
48°C
30°C
30°C
After mustard oil
90
R/s
0
Control
45nA RP
Control
45nA RP
48°C
30°C
30°C
48°C
30°C
30°C
1 min


Brush
Heat
35
Before mustard oil
Control
60nA L
Control
60nA L
R/s
0
48°C
30°C
30°C
48°C
30°C
30°C
70
After mustard oil
Control
60nA L
Control
60nA L
R/s
0
48°C
30°C
30°C
48°C
30°C
30°C
1 min

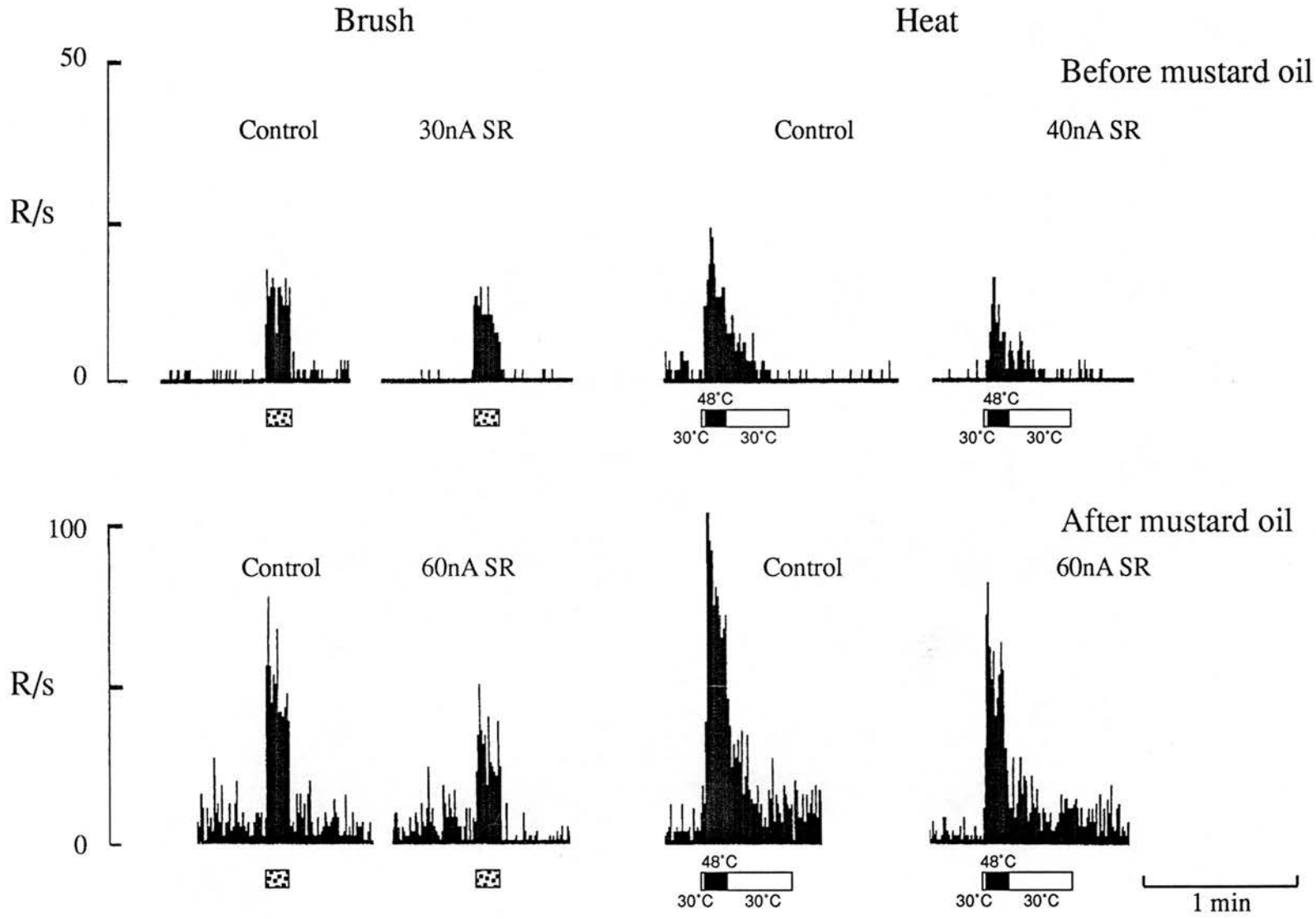

Brush
Heat
Before mustard oil
50
R/s
0
Control
30nA SR
Control
40nA SR
48°C
30°C
30°C
48°C
30°C
30°C
After mustard oil
100
R/s
0
Control
60nA SR
Control
60nA SR
48°C
30°C
30°C
48°C
30°C
30°C
1 min

Figure 4.4

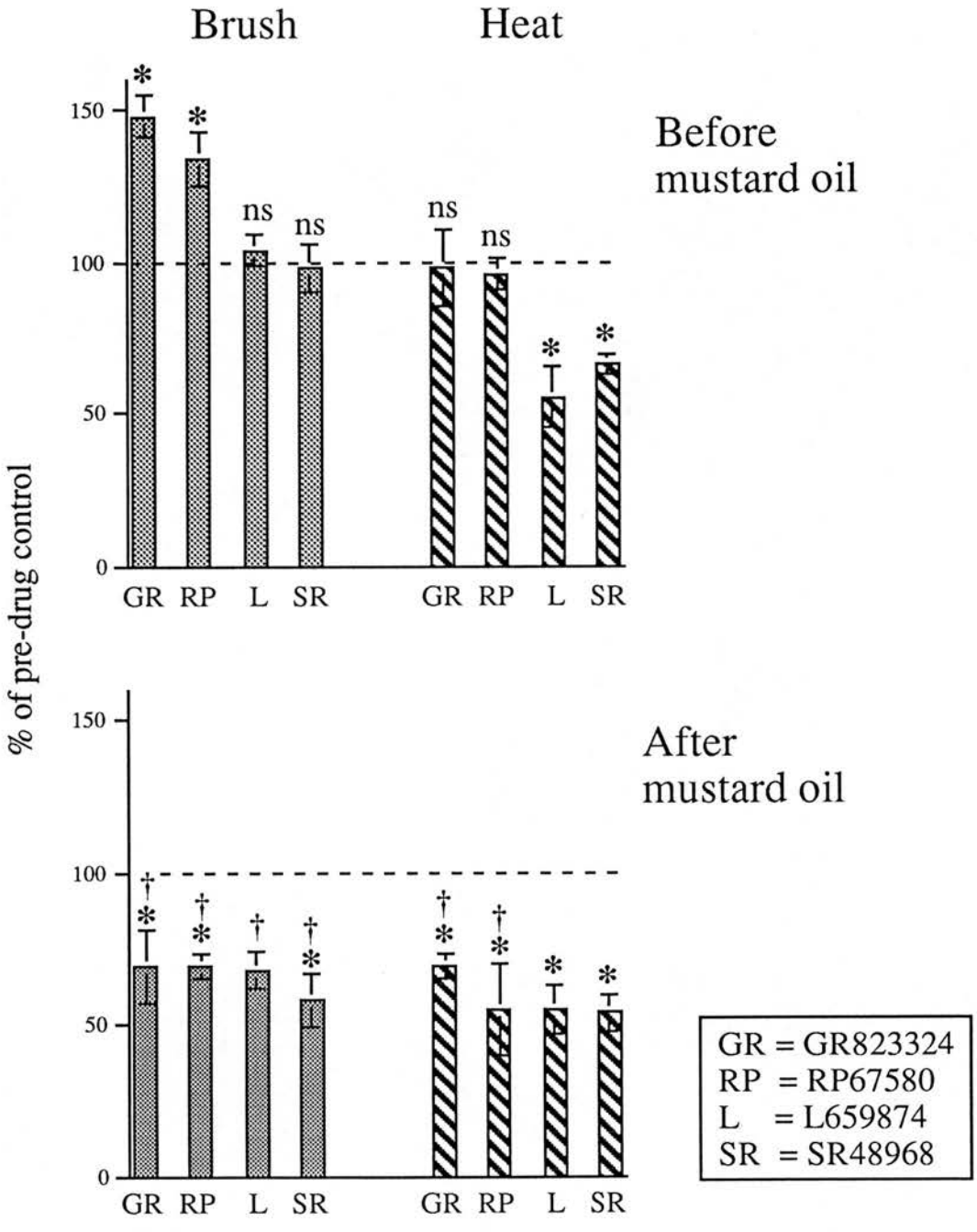
Histogram demonstrating the effect of tachykinin antagonists on sensory responses before and after cutaneous application of mustard oil.

Sensory responses to innocuous brush and noxious heat were compared before and after the peripheral application of mustard oil. The effects of the highly selective NK_1 antagonists GR 82334 and RP 67580 and NK_2 antagonists L-659,874 and SR 48968 were also tested on these multireceptive neurons both before and after mustard oil.

Before mustard oil, ionophoretic application of selective NK_1 antagonists resulted in a small, but reliable enhancement of activity evoked by innocuous brush with no apparent effect on noxious heat or spontaneous activity in 6/7 and 6/8 cases for GR 82334 and RP 67580 respectively. Similar application of NK_2 antagonists resulted in a pronounced decrease in the response to noxious heat with no significant change in spontaneous or innocuous brush activation in 8/8 and 5/5 cases for L-659,874 and SR 48968 respectively.

A very different situation resulted after peripheral sensitisation to mustard oil. The spontaneous activity increased on average by approximately 400% and ionophoresis of both NK_1 and NK_2 antagonists caused a marked decrease in both innocuous brush and noxious heat responses in this sensitised state.

There was no consistent difference in the currents required for the effects described prior to/following mustard oil. The statistical significance of drug effects compared to their concurrent control was expressed by the Wilcoxon Signed Ranks Test (* $P \leq 0.05$). The statistical significance of differences in drug effects prior to or following mustard oil was assessed by Mann-Whitney U-test.


Brush
Heat
Before mustard oil
After mustard oil
% of pre-drug control
150
100
50
0
ns
GR RP L SR
GR = GR823324
RP = RP67580
L = L659874
SR = SR48968

study produced a pronounced and significant decrease in the response to noxious heat. For L-659,874, over a current range of 15-55nA there was a 45 ± 10%, mean ± s.e.m., (n = 5) decrease and for SR 48968, a 34 ± 3%, mean ± s.e.m. decrease from control values.

(b) Post-Mustard Oil Responses

After mustard oil (8% in paraffin oil) was painted over the receptive field area on the foot and hind limb over 1-20 minutes, 23/24 cells tested had pronounced and prolonged increases in activity with firing rates at 400 ± 19% of background levels. A further 2 cells also had a high activity level after mustard oil application, but this could not be compared with pre-mustard oil spontaneous activity as this had not been recorded for technical reasons. Figure 4.1 shows a typical example of the increased firing rate, post-mustard oil. Before any sensory responses could be tested following mustard oil, it was ensured that the elevated mustard oil-induced activity had remained at a constant level for at least two minutes.

The post-mustard oil sensory responses (brush and heat) were analysed in a similar manner to the pre-mustard oil sensory responses. The values for the stimulus-evoked responses were then normalised by taking the spontaneous background value from each sensory response value and expressing as a percentage of the mean pre-drug control value.

The protocol for drug testing following mustard oil was identical to that prior to mustard oil but usually with allowance for a prolonged period of recovery from previous drugs (> 30 minutes). The results of the drug effects were compared for both before and after mustard oil application also the statistical significance of differences in drug effects prior to or following mustard oil was assessed by Mann Whitney U-Test, and a comparison with the corresponding pre- or post- mustard oil controls was made The statistical significance of drug effects compared to their concurrent control was assessed by Wilcoxon Signed Ranks Test.

The peripheral application of mustard oil to the receptive field area over a period of 5 - 20 minutes caused a sensitisation of the sensory responses compared to the pre-mustard oil control values. During this period, the receptive field area became red and slightly swollen. The responses to innocuous brush and noxious heat increased by 212 ± 27% (n=17) and 219 ± 27% (n=19) respectively reflecting the same degree of sensitisation of these responses.

The Effect of NK_1 Antagonists on Post-Mustard Oil Responses.

In the sensitised state, ionophoresis of NK_1 antagonists GR 82334 and RP 67580 produced an entirely different effect on the sensory responses, (noxious heat and innocuous brush) compared to the pre-mustard oil controls. In 7 out of 7 neurons studied, ionophoresis of GR 82334 at 25-85nA caused a marked and sustained decrease in responses to both innocuous brush and noxious heat to 69 ± 12% and 69 ± 4% respectively (mean ± s.e.m.) ($P<0.05$) (see Figures 4.1, 4.3a and 4.4). In 4 out of 4 neurons studied, ionophoresis of the non-peptide antagonist RP 67580 reproduced these effects with a decrease of 31 ± 4% (mean ± s.e.m.) in the response to innocuous brush and 45 ± 15% (mean ± s.e.m) in the response to noxious heat, expressed as a percentage of the control pre-mustard oil values (see Figures 4.2, 4.3b and 4.4). This is entirely different to the pre-sensitised state where both NK_1 antagonists produced a significant increase in the response to innocuous brush with no discernible effect on the noxious heat. These changes cannot be attributed to differences in ejection currents between pre- and sensitised state, as there was no significant difference ($P > 0.05$) between them.

In the case of GR 82334, it was possible to test sufficient neurons in a strictly matched design both before and after mustard oil, so that a precise assessment could be made of drug effect on the mustard oil-induced increment in the sensory responses, over and above any changes in basal activity (see Figure 4.1).

In these experiments, prior to mustard oil, GR 82334 had no effect on the heat responses (98 ± 12% of control, mean ± s.e.m. n = 5). However, mustard oil elicited an increase in drug-free heat responses to a mean value of 223 ± 62% of control. The increment brought about in heat responses by mustard oil was significantly inhibited by GR 82334, to a value of 16 ± 18% of the drug-free, mustard oil induced increment ($P<0.05$, by Wilcoxon test). GR 82334 had a significantly different effect on the increment in heat responses brought about by mustard oil, than it did on pre-mustard oil heat responses ($P>0.05$, Mann Whitney U-test).

Prior to mustard oil application, ionophoresis of GR 82334 (30-80nA), caused a marked and significant increase in the response to innocuous brush (to 148 ± 7% of control, $p<0.05$, by Wilcoxon test). After recovery from the NK_1 antagonist mustard oil application resulted in a significant increase in the brush response to 212 ± 27% of control (mean ± s.e.m., $P>0.05$). This increment brought about in brush responses by mustard oil was significantly inhibited by GR 82334, to a value of 69 ± 12% of the control ($P<0.05$, by Wilcoxon test).

The Effect of NK_2 Antagonists on Post-Mustard Oil Responses.

Ionophoresis of both the peptide NK_2 antagonist L-659,874 and the non-peptide antagonist SR 48968 produced similar changes in the sensory responses after peripheral application of mustard oil to the receptive field area (see Figures 4.3b,c and 4.4). In this sensitised state, L-659,874 caused a marked decrease in both brush and heat responses to 68 ± 6% (3/5 neurons) and 55 ± 8% (5/5 neurons) of the control values. Similarly, SR 48968 caused a decrease in both the innocuous and noxious responses to 58 ± 9% (4/5 neurons) and 54 ± 6% (4/4 neurons) of the pre-mustard oil control. These results are surprisingly different to those in the pre-mustard oil non-sensitised state where the NK_2 antagonists had no apparent effect on the innocuous brush, whilst inhibiting the noxious heat responses. Again this cannot be attributed to any differences in the current levels between control and sensitised states as there was no significant difference ($P>0.05$).

4.4 DISCUSSION

Stimulation of cutaneous nociceptors with mustard oil has been described to lead to central sensitisation of dorsal horn neurons and expansion of their effective receptive fields (Woolf and King, 1990; Woolf and Thompson, 1991). Central sensitisation is the phenomenon whereby neurons in the dorsal horn undergo prolonged alterations in their response properties (Woolf, 1991). It is triggered by activation of nociceptive afferents by noxious stimuli (Wall and Woolf, 1984; Woolf and Wall, 1986) or following their sensitisation, by low intensity stimuli, produce activity dependent alterations in dorsal horn neurons such that they begin to respond in an abnormal or exaggerated way to Aβ afferent inputs (Woolf and King, 1990; Woolf, 1991). The result of such central sensitised states is a prolonged reduction in the threshold, an expansion in the extent and an increased responsiveness of the cutaneous receptive fields of dorsal horn neurons (Campbell *et al*, 1979; LaMotte *et al*, 1983; Cervero *et al*, 1988; Hylden *et al*, 1989; Simone *et al*, 1991; Woolf and King, 1990). Although NK_2 rather than NK_1 receptors seem important in mustard oil-evoked increases in spontaneous activity here, it is not clear whether NK_1 receptors may play a role in central sensitisation.

The present experiments thus specifically addressed the role of NK_1 and NK_2 receptors in central sensitisation. The neuronal population sampled here had

similar electrophysiological characteristics and receptive field locations as the cells reported in Chapter 3. Only multireceptive neurons were studied because of their ability to integrate both noxious and innocuous information (Iggo, 1974; Handwerker *et al*, 1975). Their position was substantiated by depth measurements and blue spot histology and correspond to laminae III-V of dorsal horn as defined in rats by Molander *et al*, (1984). Control studies demonstrated that whilst DLH ejection caused clear excitation, ejection of NaCl and vehicle were unable to reproduce any of the observed actions described below. The cutaneous receptive field of each neuron under examination was located on the 'hairy' foot, toes or flank of the ipsilateral hindlimb. Neurons with receptive fields on the glabrous skin were not used due to poor penetration of the chemical algogen mustard oil (Harris and Ryall, 1988).

Ionophoresis of the highly selective NK_1 and NK_2 antagonists around the laminae III-V dorsal horn neurons produced results entirely consistent with those obtained in Chapter 2. The selective peptide and non-peptide NK_1 antagonists used were GR 82334 (Hagan *et al*, 1991) and RP 67580 (Garret *et al*, 1991) respectively. Local application of each drug around laminae III-V dorsal horn neurons resulted in a consistent facilitation of the response to innocuous brush with no apparent change in the sensory responses to noxious pinch and heat. Also analogous to Chapter 2 are the consequences after ionophoretic administration of the highly selective NK_2 antagonists L-659,874 (M^{c}Knight *et al*, 1988) and SR 48968 (Emonds-Alt *et al*, 1992), a selective attenuation of the noxious heat response with no significant effect on innocuous brush or noxious pinch.

Following repeated cutaneous application of mustard oil to the neuronal receptive field, there was a significant, facilitated increase in the background firing rate. As described in Chapter 3, some cells had an immediate increase in firing rate after a single application of this chemical algogen, however, in most cases, the steady elevated firing rate was achieved by repeated application of the algogen. However, a crucial dissimilarity between the experiments carried out here and those in Chapter 3 was the length of time each neuron was exposed to mustard oil before ionophoresis of selective antagonists. As each set of brush and heat responses were cycled over a minimum period of 3 minutes and at least three consecutive sets of control sensory responses were carried out here before drug testing, then the latency between the initial mustard oil application and the ionophoresis of selective antagonists was on average much greater.

This prolonged exposure to the selective C fibre stimulant was critical to the responsiveness of each neuron as consequently the responses to both

innocuous brush and noxious heat were markedly and significantly increased taking background activity into account. Furthermore, consistent monitoring of the receptive field size throughout each experiment revealed that whereas in the last series of experiments there was no observed differences consequent to mustard oil exposure, here an increase was frequently observed suggestive of the involvement of a central alteration (Woolf, 1991). This may be simply due to the increased exposure to mustard oil, although another possible explanation is the application of repeated control sensory responses to a mustard oil-facilitated neuron. Although in a non-mustard oil treated condition, cycling of the brush and heat sensory responses at 3 minute intervals has never resulted in an observed sensitisation of the dorsal horn neurons studied (Fleetwood-Walker *et al*, 1990), perhaps in a system which has been 'primed' by cutaneous application of mustard oil this can easily occur.

Correspondingly, in this 'sensitised' state, the balance of NK_1 and NK_2 receptor involvement in the sensory responses to innocuous brush and noxious heat appeared to be altered. Although ionophoresis of both the highly selective NK_2 antagonists L-659,874 and SR 48968 resulted in a selective attenuation of the response to noxious heat in both the pre-mustard oil and sensitised states, in the sensitised state there was additional inhibition of the facilitated innocuous brush sensory response. Furthermore, administration of selective NK_1 antagonists had different consequences in each case. Whereas in the pre-mustard oil state, ionophoretic administration of both GR 82334 and RP 67580 close to the laminae III-V neurons resulted in a marked and selective facilitation of the response to innocuous brush with no significant effect on noxious heat responses; in the sensitised state the facilitation of neuronal responses to both brush and heat was blocked by the NK_1 antagonists.

Interest was primarily in the influence of the selective tachykinin antagonists on the cutaneous sensory responses in this 'sensitised' state and in general any corresponding effect on the background activity was not calculated. However, from the examples of neuronal activity shown in Figures 4.1, 4.2 and 4.3 it can be seen that although the mustard oil-facilitated background activity was low, following ionophoretic administration of both selective peptide and non-peptide NK_1 (GR 82334 and RP 67580 respectively) and NK_2 receptor antagonists (L-659,874 and SR 48968 respectively), there was attenuation of the facilitated background activity for each example. These results were in contrast to those described in Chapter 3 whereby although ionophoresis of the selective peptide and non-peptide NK_2 receptor antagonists L-659,874 and SR 48968

resulted in a similar, marked attenuation of the mustard oil-facilitated background activity in both series of experiments, the results obtained following ionophoretic administration of selective NK_1 antagonists was entirely different. In Chapter 3 following acute nociceptive chemical stimulation of dorsal horn neurons, the mustard oil-facilitated background was generally unaltered following ionophoretic administration of selective peptide (GR 82334 and L-668,169) NK_1 receptor antagonists but in a few examples where the selective non-peptide NK_1 receptor antagonist RP 67580 was either ionophoretically or intravenously given, there was attenuation of the amplified response to mustard oil.

These differences are likely to be due to the development of sensitisation following the prolonged exposure of the neurons to mustard oil. Although the background activity was sometimes very low following prolonged exposure to the algogen, the fact that the receptive field size increased and the sensory responses were facilitated, demonstrated that after prolonged application of the C fibre stimulant mustard oil to a cutaneous receptive field, central sensitisation occurred, in agreement with previous studies using mustard oil (Woolf and Wall, 1986; Woolf and King, 1990) and also the C-fibre neurotoxin capsaicin (Simone *et al*, 1991; Dougherty and Willis, 1992; LaMotte *et al*, 1992). Central sensitisation is a phenomenon known to occur following activation of nociceptive afferents by sustained noxious or inflammatory stimuli that lead to hyperalgesia and allodynia resulting in changes in the excitability of the spinal cord dorsal neurons (Woolf, 1991; Treede *et al*, 1992). In close agreement with the results described, central sensitisation is characterised by a reduction in threshold, an increased responsiveness of dorsal horn neurons to thermal (Kenshalo *et al*, 1979; Simone *et al*, 1991) and mechanical stimuli (Kenshalo *et al*, 1979; Simone *et al*, 1991; Woolf and King, 1990) and an expansion in the extent of the receptive field (Treede *et al*, 1992). Many of these changes (especially secondary hyperalgesia outwith the primary zone of injury) cannot be adequately explained by peripheral changes, and therefore must be due to central sensitisation (Woolf, 1991; Treede *et al*, 1992).

It therefore is apparent that although NK_1 receptors appear to be less involved in acute nociception, they may have a role in mediating 'sensitised' nociceptive responses. Consistent with this idea, inflammatory models of sustained nociception induced by formalin and CFA have been shown to increase the biosynthesis of SP-IR and mRNA encoded by the PPT-1 gene in both the spinal dorsal horn and dorsal root ganglia of rats (Minami *et al*, 1989; Donaldson *et al*, 1992; Noguchi and Ruda, 1992; McCarson and Krause, 1994). Following

induction of these inflammatory states, an increase in the NK_1 receptor gene expression and NK_1 binding sites have also been observed (Schafer *et al*, 1993; Stucky *et al*, 1993; McCarson and Krause, 1994). Furthermore, 'sensitising' models of sustained C-fibre activity have demonstrated the increased release of SP-IR into the spinal dorsal horn following a potential damaging thermal stimulation (Go and Yaksh, 1987; Kuraishi *et al*, 1989) in addition to the more prolonged inflammatory models of formalin, CFA or carrageenan/kaolin injection (Oku *et al*, 1987; Kuraishi *et al*, 1989; Schaible *et al*, 1990; McCarson and Goldstein, 1991; Garry and Hargreaves, 1992).

In addition, repetitive electrical stimulation of afferents at intensities sufficient to activate C-fibres, correspondingly elicits the sensitised responsiveness of dorsal horn neurons that is known as 'wind-up' (Mendell, 1966) and is thought to contribute to central hyperalgesic states (Woolf, 1983; Dickenson, 1990; Woolf and Thompson, 1991). Accordingly, SP was able to enhance, whilst a NK_1 receptor antagonist was able to inhibit the responses of nociceptive dorsal horn neurons to wind-up (Kellstein *et al*, 1990), in addition to the underlying long-duration excitatory postsynaptic dorsal root potentials (Urban and Randic, 1989). Furthermore, using an *in vitro* spinal cord-skin preparation, these long-duration epsps have also been recorded extracellularly as ventral root potentials in the recently developed ultraviolet (UV) burn hyperalgesic model. It was demonstrated that the NK_1 receptor antagonist CP,96,345 was able to reduce C-fibre evoked ventral root potentials in the UV-treated state yet was ineffective in naive rats (Thompson *et al*, 1993) demonstrating a role for NK_1 receptors in this model of central sensitisation. In addition, the facilitation of the flexion-reflex induced by C-fibre strength conditioning stimulation to the sural nerve, believed to lead to central sensitisation, was mimicked by intrathecal administration of SP (Woolf and Wiesenfeld-Hallin, 1986; Wiesenfeld-Hallin *et al*, 1990; Xu *et al*, 1992) and effectively antagonised by selective NK_1 receptor antagonists (Wiesenfeld-Hallin *et al*, 1990; Xu *et al*, 1992; Laird *et al*, 1993). However, the baseline reflex remained unaffected following pre-treatment with NK_1 receptor antagonist (Laird *et al*, 1993).

Behavioural studies have further substantiated a role for NK_1 receptors in sustained or inflammatory nociceptive models. The reduction in tail-flick latency following sensitisation of the rat's tail in hot water can be reversed following NK_1 receptor antagonist (Cridland and Henry, 1988; Yashpal *et al*, 1993). Similarly, both the NK_1 receptor antagonists RP 67580 and CP-96,345 were antinociceptive in the formalin model of inflammation and hyperalgesia (Garrett *et al*, 1991;

Yamamoto and Yaksh, 1991; Birch *et al*, 1992; Nagahisa *et al*, 1992; Chapman and Dickenson, 1993; Yashpal *et al*, 1993) although there are reports of non-NK_1 receptor mediated effects (Nagahisa *et al*, 1992; Rupniak *et al*, 1993). Likewise, the carrageenan-induced oedema and hyperalgesia can be attenuated following (±)CP-96,345 (Birch *et al*, 1992) although again, spurious non-NK_1 receptor-mediated effects have been suggested (Nagahisa *et al*, 1992). However, CP-96,345 has been shown to stereospecifically inhibit mustard oil-evoked oedema and acetic acid-induced writhing (Lembeck *et al*, 1992; Nagahisi *et al*, 1992) further substantiating a role for NK_1 receptors in inflammatory and hyperalgesic conditions.

In the periphery, there is further evidence suggesting that NK_1 receptor effects are highlighted following induction of inflamed or sustained nociceptive conditions. The non-peptide receptor antagonists RP 67580 and CP-96,345 dose-dependently reduced plasma extravasation produced by electrical stimulation of the trigeminal ganglion (Shepheard *et al*, 1993), intravenous injection of SP (Lembeck *et al*, 1992; Nicolau *et al*, 1993; Moussaoui *et al*, 1993) or antidromic sciatic nerve stimulation (Xu *et al*, 1992). Moreover, brief capsaicin treatment of rabbit skin nerves leads to a reduction in the neurogenic, inflammatory response to mustard oil and also in the SP content of the skin (Lynn and Shakhanbeh, 1988) further supporting a role for SP in plasma extravasation and inflammation.

In contrast to the NK_1 receptors, NKA (and corresponding NK_2 receptors) clearly play a functional central role in, brief thermal nociceptive responses (see Chapter 2); medium-term sustained C-fibre activity following acute cutaneous application of mustard oil (see Chapter 3); in addition to the sensitised neuronal responses to brush and heat following prolonged exposure to the chemical algogen mustard oil. It is therefore apparent that, unlike NK_1 receptors which appear to only be functional in inflammatory and hyperalgesic conditions, NK_2 receptors play a role in mediating models of both acute and sustained, inflammatory pain. However, following central neuronal sensitisation, this role seems to be modified with NK_2 receptors mediating both noxious and innocuous neuronal responses. In support of the role of NK_2 receptors in mediating sustained, sensitised spinal nociceptive processing, other studies have shown that adjuvant or formalin-induced inflammation and hyperalgesia leads to an increased biosynthesis of PPT-1 mRNA (and therefore possibly NKA too) (Minami *et al*, 1989; Donaldson *et al*, 1992) and although there is as yet no direct evidence for an increase in NK_2 receptor biosynthesis due to inflammation and hyperalgesia, increased release of NKA was seen following kaolin and carrageenan injection

into the knee joint (Hope *et al*, 1990). Furthermore, molecular biology techniques have also been utilised to provide powerful evidence that NK_2 receptors play a role in longer-term genomic responses to inflammation. Using *in situ* hybridisation histochemistry, the contribution of NK_2 receptors to the carrageenan-induced expression of preprodynorphin mRNA in superficial dorsal horn neurons was assessed (Parker *et al*, 1993). Locally applied NK_2 antagonists effectively reduced the response to carrageenan, both in terms of number of expressing cells and density of expression. Electrophysiological studies provide further support for a role of NK_2 receptors in mediating sensitised responses, the flexor reflex facilitation evoked by damaging cutaneous thermal stimuli (80°C for 0.5 seconds) was significantly potentiated by intrathecal NKA (Xu and Wiesenfeld-Hallin, 1992). Likewise, pre-treatment with the selective NK_2 receptor antagonist, MEN 10207 effectively blocked the long-term flexor reflex facilitation due to gastrocnemius nerve conditioning at C fibre strength (Xu *et al*, 1991). Correspondingly, in the ultraviolet burn model using an *in vitro*, spinal cord-skin preparation (Thompson *et al*, 1994), the selective NK_2 antagonist MEN 10376, significantly reduced the C-fibre evoked ventral root potential both pre- and post-inflammation, again providing evidence that NK_2 receptors are important in both acute and prolonged nociceptive states.

It is not yet clear whether the role in sensitisation of NK_1 receptors that emerges after inflammation is simply due to extra production, output and spread of SP to NK_1 sites some way from the release site or whether some distinct enabling signal has been received which permits the receptors to modify cellular function. Their influence, however, extends beyond the mediation of nociceptive input since they also sensitise with respect to non-nociceptive inputs, emphasising an effective role as sensitiser rather that just input mediator. Whilst a full elucidation of these regulatory mechanisms presents a considerable research task, it is possible, with relative ease, to identify NK_1 and other receptors from such experiments as being viable therapeutic targets.

CHAPTER 5:
The Role of Protein Kinase C (PKC) In Sustained Nociceptive Responses of Rat Dorsal Horn Neurons

5.1 AIMS

The present experiments addressed any role of protein kinase (PKC) in sustained nociceptive responses of rat dorsal horn neurons by (i) ionophoretic administration of PKC inhibitors whilst recording activity evoked by repeated cutaneous application of mustard oil; and (ii) assessing subcellular translocation of PKC evoked in spinal cord by cutaneous application of mustard oil. In addition, the involvement of NK_2 receptors was assessed using systemic administration of selective antagonists.

5.2 MATERIALS

Animals: Male Wistar rats were obtained from Charles River UK Ltd, Margate, Kent, UK.

Anaesthetics: Alpha-chloralose and urethane were obtained from Sigma Chemical Company, Poole, Dorset, UK; Halothane (flurothane) was obtained from ICI Pharmaceuticals, Macclesfield, Cheshire, UK.

Laboratory Chemicals: Standard laboratory chemicals were of Analar grade and from the following suppliers: Sodium chloride (NaCl), DLH (D, L, homocysteic acid) and Pontamine Sky Blue (PSB) were obtained from Sigma Chemical Company, Poole, Dorset, UK; allyl-isothiocyanate (mustard oil) was purchased from Aldrich Chemical Company Ltd, Gillingham, Dorset, UK; Halothane (Flurothane) was obtained from ICI Pharmaceuticals, Macclesfield, Cheshire, UK. Agar was obtained from Oxoid Ltd, Basingstoke, Hampshire, UK and paraffin was purchased from Scotlab, Lanarkshire, UK. [20-^{3}H(N)]-phorbol,12,13-dibutyrate ([^{3}H]-PDBu) (specific activity 19.1 Ci/mmol) was supplied by Du Pont, Dreiech, Germany. Bovine serum albumin (essential fatty acid free), PDBu, 2-mercaptoethanol and phosphatidylserine were all purchased from Sigma Chemical Company Ltd, Poole, Dorset, UK.

Drugs: The protein kinase C inhibitors, chelerythrine and 2-[-(3-dimethylaminopropyl)-indol-3yl]-3-(-indol-3yl)maleimide, (GF109203X) were obtained from Novabiochem, Nottingham, UK); (S)-N-methyl-N[4-(4-

acetylamino-4-phenyl piperidino)-2-(3,4-dichloro phenyl)butyl] benzamide (SR 48968) and its (R) enantiomer SR 48965, were gifts from Dr X Emonds-Alt, Sanofi Recherché, France.

5.3 METHODS:- ELECTROPHYSIOLOGY

5.3.1 General Methods

See Chapter 2 for details of animals; surgical techniques; procedures for histological determination of laminar location and recording & ionophoresis methods used. Extracellular recordings from single laminae III-V dorsal horn neurons, were made through the central barrel of a 7-barrelled microelectrode containing 4M NaCl, pH 4.0-4.5. Drugs were ionophoresed from the side barrels. One side barrel contained 1M NaCl (pH 4.5-5.0) for automatic current balancing, another barrel was filled with PSB for marking the position of recording sites and the other electrode barrels contained the following selective PKC inhibitors:- Chelerythrine (1mM, pH 4.5-5.0); GF 109203X (0.2mM in 0.2% DMF, pH 4.5-5.0).

5.4 METHODS:- [^{3}H]PHORBOL 12,13-DIBUTYRATE BINDING STUDIES

5.4.1 Animals

Adult male Wistar rats (250-360g) were housed in groups of 3 or 4 in standard plastic cages, maintained on a 12 hour light to 12 hour dark cycle at 22-23°C; food and water available ad libitum throughout the experiments.

5.4.2 Drugs

The non-peptide NK_2 receptor antagonist SR 48968 and the inactive isomer SR 48965 were used in these experiments. Each was dissolved in a few drops of ethanol, then saline (0.9% NaCl) to give 1mg/ml solutions and injected as a 1.0 mg/kg solution by an intraperitoneal (i.p.) route. Both drugs were made fresh on the day of the experiment and were not frozen or reused.

5.4.3 Experimental Protocol

If an antagonist or vehicle was under study, the rats were injected by the i.p. route 15 minutes prior to administration of anaesthetic, during which time they remained in the cage with free access to both food and water.

Figure 5.1 shows a summary diagram of the methods employed. The rats were lightly anaesthetised with flurothane and both hind legs were shaved. Mustard oil (allyl isothiocyanate) was diluted to 8% in paraffin oil and painted onto the left hind-paw and limb using a paintbrush and covering up to the knee joint. The stopwatch was started and at 5 and 10 minutes this procedure was repeated. After the final application of mustard oil, the treated limb was carefully covered with cling film and tape to avoid any cross-contamination of the contralateral limb. The rat was placed in a restraining cage made from wire mesh and cushioned with foam. It was then left to recover from the anaesthetic and during this period the animal was closely monitored to ensure that the bandage did not come off.

At 30 minutes post-mustard oil treatment, the animal was killed by cervical dislocation and the spinal cord was removed as rapidly as possible, within 2-4 minutes. Following a laminectomy using sterile dissection instruments, the T12-L6 area was exposed and the dura was carefully removed to allow easier removal of the cord. Using a sterile surgical blade (No. 22), the cord was very carefully hemisected along the midline and most of the dorsal roots were removed; only the L2-L5 area was taken for the assay.

Each half of the cord was separately homogenised in 450µl of ice-cold buffer (20mM Tris-HCl (pH 7.5) with 50mM 2-mercaptoethanol, 2mM EDTA and 1mM phenylmethylsulphonyl fluoride) containing 0.01% leupeptin and 20µM transepoxysuccinyl-L-leucylamido-(4-guanidino)- butane (E-64) ("Kuo-PI") using hand-held 1ml glass homogenisers. The samples were than ultracentrifuged (38,000 x g , 20min, 4°C) to separate membranes and cytosol. The cytosolic fractions (supernatant) were carefully removed using fine glass pipettes and equilibrium ligand binding assays carried out as follows:-

The membranes were resuspended, using an Ystral polytron homogeniser, in 300µl 50mM Tris HCl (pH 7.4) with 4mg/ml bovine serum albumin (essential fatty acid-free) (BSA). The membrane suspension (50µl) was aliquoted into tubes on ice containing: 200µl 50mM Tris HCl (pH 7.4) with 4mg/ml BSA; 25µl of [^{3}H] PDBu and 2.5µl dimethylformamide or 2.5µl of 1mM PDBu in dimethylformamide for measurement of total or non-specific binding levels respectively. Tubes were capped, vortexed and placed in a waterbath at 37°C for 30 minutes. The tubes were then placed on ice and protein was precipitated by addition of 300µl of 'Tris-PEG' (50mM Tris-HCl (pH 7.4) with 24% polyethylene glycol 8000 and 100µl of 'Tris-BGG' (50mM Tris-HCl (pH 7.4) with 12mg/ml of

Figure 5.1

Summary diagram of the procedures used in [^{3}H]phorbol ester binding studies in the rat spinal cord.

Male rats were anaesthetised and mustard oil (8%) applied 3 x 5 min intervals to an extensive area of shaved skin distal from the thigh on one hindlimb. After 30 minutes, spinal segments L2-L5 were carefully removed and hemisected on the midline for analysis. Tissue was homogenised and centrifuged before specific binding of [^{3}H]PDBu was determined as outlined.

[^{3}H]PDBu Binding In Rat Spinal Cord.

Adult male rat.

↓

Anaesthetised, both legs shaved.

↓

Left hindpaw and foot painted with 8% M Oil 3x over 15 minutes.

↓

Treated leg bandaged to prevent cross-contamination.

↓

Rat transferred to restraining cage for 30 mins.

↓

Rat killed, spinal cord hemisected.

↙ ↘

Left (treated) hemisection | Right (control) hemisection

↘ ↙

Each hemisect separately homogenised in 400µl ice-cold buffer (20mM Tris-HCL (pH 7.5) with 50mM 2-mercaptoethanol, 2mM EDTA & 1mM phenylmethylsulphonyl fluoride) containing 0.01% leupeptin and 20µM transepoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64).

↓

Ultracentrifuged 250,000g, 30 mins 4°C.

↙ ↘

Supernatant 50µl/tube

↓

Additons to each tube

200µl 1.25mg/ml PS in buffer (containing 50mM Tris HCl, 4mg/ml BSA, 1mM $CaCl_2$, 75mM Mg Acetate); 25µl ^{3}H PDBu in TBSA (10µl/ml); 2.5µl DMF (total) or 2.5µl PDBu (non-specific)

↘

Membranes

Pellet resuspended in Tris 50mM HCL Fatty Acid Free (FAF) Bovine Serum Albumin (BSA) 4mg/ml in pH 7.0 50µl/tube

↓

Additons to each tube

200µl TBSA; 25µl ^{3}H PDBu in TBSA (10µl in 1 ml); 2.5µl DMF (total binding) or 2.5µl PDBu (non-specific).

↙

Cap, vortex 37°C 30 mins.

↓

Proteins precipitated on ice with 12mg/ml bovine gamma globulin and 24% polyethylene glycol 8000

↓

Vortex.

↓

Ice, 20 mins.

↓

Centrifuge 12,000g, 5min 4°C.

↓

Tips cut from tubes.

↓

Count.

bovine gamma globulin (BGG)) to each tube. The tubes were capped, vortexed and left on ice for 20 minutes before being centrifuged (12,000 x g, 5 minutes, 4°C) and the supernatant aspirated. The tip of each tube was cut off and left to dissolve overnight in 4ml scintillation fluid (Emulsifier-Safe, Packard Ltd., Groningen, Netherlands). The radioactivity in each pellet was determined by counting in a Beckman LS 1801 liquid scintillation counter.

The cytosolic [^{3}H]PDBu binding assay was performed in a method similar to that described by Leach *et al,* (1983). The supernatant removed after ultra-centrifugation was regarded as the cytosolic fraction, 50µl was aliquoted into tubes on ice containing 200µl of a micellar preparation of phosphatidylserine (sodium salt) in assay buffer (1.25 mg/ml final concentration), 25µl [^{3}H]PDBu (10 µl/ml final concentration) and 2.5µl dimethylformamide or 2.5µl of 1mM PDBu in dimethylformamide for measurement of total or non-specific binding levels respectively. The assay buffer ("Blumberg cytosol buffer") contained 50mM Tris HCl; 4mg/ml BSA, 1mM $CaCl_2$; 75mM magnesium acetate. Phosphatidyl serine micelles were prepared by sonication, assay tubes capped, vortexed and incubated at 37°C for 30 minutes. Protein was precipitated on ice by addition of 100µl of 12 mg/ml bovine gamma-globulin and 300µl of 24% polyetheneglycol 8,000 in 50mM Tris HCl (pH 7.4), capped and vortexed vigorously. After 20 min (4°C), assay tubes were spun (12,000 x g, 5 min, 4°C), aspirated and the radioactivity in each pellet was determined by solution overnight in scintillation fluid, then counting as described above for membrane assays.

5.4.4 Analysis

After each hemisected piece of spinal cord had been homogenised and centrifuged, the resulting cytosol and membrane components were each transferred into 4 tubes. Thus for each of cytosolic and membraneous components there were 2 tubes for measurement of total binding and a further 2 for non-specific binding measurement. After completion of the assay, each tube was counted for β-radiation and the results were expressed graphically (see Figures 5.3 and 5.4). Each pair of results were averaged, and the difference between the mean total and mean non-specific binding was regarded as the specific binding.

The subcellular translocation of [20-^{3}H(N)]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding sites from cytosolic to membrane compartments, can be taken as an index of PKC activation in response to stimulation of cell surface receptors (Lutz *et al,* 1993). Thus by comparing the cytosolic and membrane

components from each hemisected piece of cord, it could be calculated whether PKC translocation / activation had occurred.

Statistical significance of changes was assessed by Mann-Whitney U-test. This is a nonparametric statistical analysis test which makes no assumptions of a normal distribution of data and errors. It is therefore a convenient statistical test to analyse both raw data, and data which is transformed (e.g., ratioed to some control value) as is mostly presented here. Although the Mann-Whitney U-test is not the most powerful test to detect small differences, it does demand the constraints on data distribution of the Student's t-test, which can be violated easily in practice. To apply the test, all the data have to be assigned a rank order of magnitude (e.g. number 1 for lowest value to number n for the highest value (n = total number of values in both data groups being compared)). The rank orders in both groups are summed, and the statistical significance for the appropriate number of values in each group, can be determined using a Mann-Whitney or Wilcoxon significance table. Significance was accepted at probability values of $P \leq 0.05$.

5.5 RESULTS- ELECTROPHYSIOLOGY

5.5.1 Characteristics and Receptive Field Properties of Neurons Tested

The present results were obtained from 11 neurons located in laminae IV-V in the spinal cord dorsal horn. Laminar location was estimated as the depth recording on the microdrive which for laminae IV-V was 350-600μm. Very occasionally recording took place from cells located slightly superficial to this depth but recording from cells deeper than 600μm was very uncommon due to a tendency for unresponsiveness to the chemical algogen mustard oil at this depth. Upon cessation of recording each cell was marked by ejection of Pontamine Sky Blue (PSB) for 10-15 minutes and when the experiment terminated, the spinal cord was removed and histologically treated to enable identification of the recording electrode position on transverse sections of the cord (see Figure 2.5).

All the neurons studied had cutaneous receptive fields located on the hairy side of the foot and toes or the flank of the ipsilateral hindlimb. It was important that the receptive field was not on the glabrous skin of the plantar surface of the foot as there was poor penetration by mustard oil on this type of skin and the result was that the neuron under examination was not activated by the algogen. The neurons were initially identified by widespread manual brushing over the ipsilateral hindlimb and foot; the receptive field was then mapped with an innocuous brush stimulus and the receptive field area marked with waterproof ink.

The size of the receptive field area varied considerably between neurons, from as small as a single digit to the whole flank of the ipsilateral limb and lower body; throughout the experiment the receptive field size was continually monitored and any changes were noted.

All the neurons were multireceptive (responded to both innocuous and noxious stimuli). It was particularly important that the neuron had a notably strong response to noxious heat as any neurons tested without this were generally not activated by mustard oil. Heat-evoked sensory responses are derived mainly from C fibres with threshold skin-surface temperature usually in the range of 44-50°C (Perl, 1984) however, temperatures above 48°C are generally considered to be damaging (Duggan *et al*, 1988) and evidence has been presented that mustard oil elicits activation of largely C afferents (Woolf and Wall, 1986; Heapy *et al,* 1987), although it is possible that some Aδ activation also occurs transiently (Harris and Ryall, 1988). Generally before application of the mustard oil, the background activity of the neurons examined was very low (less than 1Hz). If the background activity was too high before application of mustard oil then it masked the facilitatory response and so these neurons were not used.

Ionophoresis of D,L homocysteic acid (DLH) was used to ensure that any drug responses were not due to movement of the recording electrode relative to the neuron. The response to ionophoresis (at the same current) of DLH remained constant throughout the experiment.

5.5.2 Current and Vehicle Controls

Neurons under consideration were routinely tested with vehicle or current controls. They were examined under identical conditions to the inhibitors under study with ejection for at least 1 minute increasing the current in a 10-20nA stepwise manner every consecutive minute. The effect of vehicle (0.2% DMF) or NaCl at up to 80nA was investigated in 3 neurons. In 3 out of 3 cells tested, ionophoresis of vehicle or NaCl at up to 80nA had no detectable effect on neuronal responses to cutaneous stimuli.

5.5.3 Effect of Cutaneous Application of Mustard Oil

The receptive field area of the neuron under study was characterised manually with a soft paintbrush and mapped out with waterproof ink. After a 8% solution of mustard oil (allyl isothiocyanate) in paraffin oil was painted onto the restricted area, all the multireceptive neurons tested (9/11) had a large and prolonged increase in activity with firing rates of 324.3 ± 75.8 fold of background

(mean ± S.E.M.) which remained sustained for a minimum period of 5-6 minutes after peripheral application.

Similar to the results achieved in Chapter 3, there were two general patterns of mustard oil-induced activation; either an immediate increase in firing rate after application of the algogen or, more commonly, the steady elevated firing rate was achieved by repeated application of mustard oil to the receptive field.

Records such as those shown in Figure 5.2 were analysed off-line to calculate the percentage inhibition by the protein kinase C inhibitors of the mustard oil increased baseline. It was important to ensure that the elevated mustard oil-induced activity had remained at a constant level for at least 2 minutes before ionophoresis of the inhibitors. To calculate the drug-induced inhibition of mustard oil-facilitated baseline activity, the number of mustard oil- induced action potentials in 3 separate 30 second periods was integrated and an average value calculated (taking spontaneous activity into account) as a percentage of the corresponding mean pre-drug mustard oil baseline value. Statistical significance was calculated using the Wilcoxon test.

5.5.4 Effect of Ionophoretically Applied Protein Kinase C Inhibitors

The effects of two ionophoretically-applied PKC inhibitors ejected close to multireceptive dorsal horn neurons in laminae IV-V of the spinal cord were assessed. The highly selective PKC inhibitors used were chelerythrine (Herbert *et al,* 1990) and GF109203X (Toullec *et al,* 1990) which were tested first on mustard oil-facilitated background activity and secondly on continuous brush activity (see Figure 5.2).

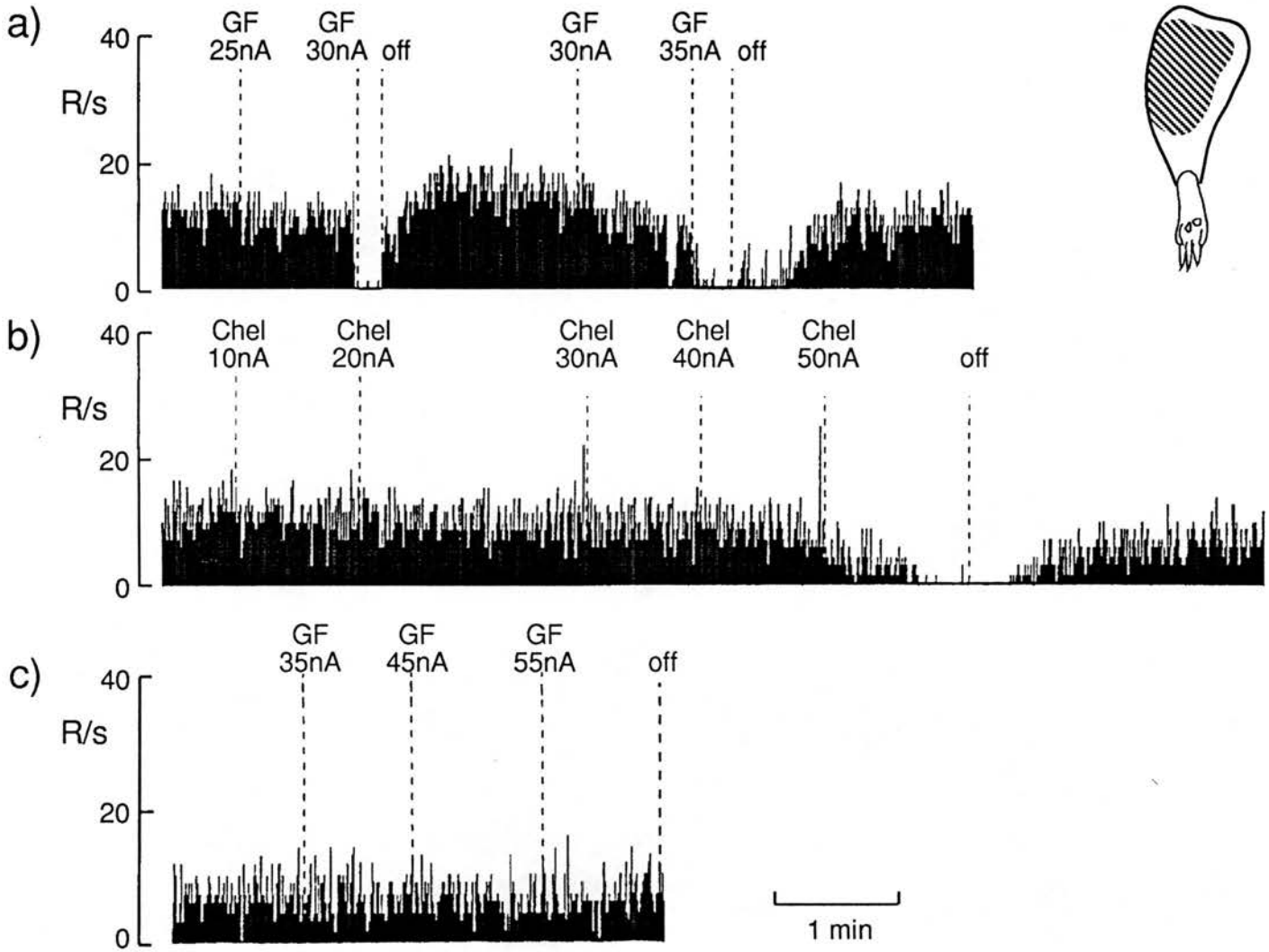
(a) Effect of Protein Kinase C Inhibitors on Mustard Oil-Induced Activity

The PKC antagonists profoundly inhibited the mustard oil evoked activity of dorsal horn neurons, generally within 1 minute of ionophoresis (see Figure 5.2 for a typical example). In 8 out of 9 cells tested with GF109203X at 20-60nA, marked inhibition of the mustard oil-evoked activity was seen (58 ± 9% inhibition (mean ± s.e.m.)). Similarly in 8 out of 8 cells tested with chelerythrine at 10-80nA, mustard oil evoked activity was inhibited by 70 ± 10% (mean ± s.e.m.).

Figure 5.2

Typical effects of protein kinase C (PKC) inhibitors, chelerythrine and GF 109203X on stimulus-evoked activation of multireceptive dorsal horn neurons.

Continuous records of firing frequency (responses/s; R/S, bin width 1000 ms) are plotted against time. Prior to testing, all neurons examined showed a low spontaneous firing rate (<0.1 Hz). Activity was evoked in (a) and (b) by mustard oil (8% in paraffin oil) applied to the receptive field 4 x 5 min intervals until a steady elevated firing rate was maintained or in (c) by motorised brush applied continuously to an equivalent receptive field without mustard oil treatment. Drugs were applied ionophoretically at the indicated currents (maintained until the subsequent marker) and at all other times were subject to a retention current of -10 nA. Results are typical of 8 out of 8 cells and 4 out of 5 cells tested with GF 109203X (GF) in (a) and (c) respectively, and of 8 out of 9 cells tested with chelerythrine (Chel) in (b).


a)
GF
25nA
GF
30nA
off
GF
30nA
GF
35nA
off
R/s
40
20
0
b)
Chel
10nA
Chel
20nA
Chel
30nA
Chel
40nA
Chel
50nA
off
R/s
40
20
0
c)
GF
35nA
GF
45nA
GF
55nA
off
R/s
40
20
0
1 min

(b) Effect of Protein Kinase C Inhibitors on Continuous Brush-Induced Activity.

A continuous brush activity was maintained for a minimum period of 1 minute before ionophoresis of the highly selective PKC inhibitors was initiated. The drugs were ionophoresed for minute at each current and the ejection current increased by 10-20nA in a stepwise manner until there was a drug-induced effect.

In 4 out of 5 cells where GF109203X was tested on continuous brush-evoked activity, there was no discernible effect, with only a small inhibition (<15%) in the fifth cell.

5.6 RESULTS - PDBU BINDING STUDIES

5.6.1 The Effect of Mustard Oil on PDBu Binding in Rat Spinal Cord.

Tissue was successfully obtained from 9 preparations and demonstrated a marked translocation of [^{3}H]PDBu binding sites from cytosolic to membrane fractions on the side ipsilateral to mustard oil compared to contralateral control (Figure 5.3). For each hemisected piece of cord, the specific binding i.e. dpm per assay tube (total minus non specific binding) was calculated for both membrane and cytosolic fractions. The percentage of the recovered specific binding found in the membrane fraction (i.e. specific binding in the membrane fraction/ specific binding in membrane fraction + specific binding in cytosolic fraction) was calculated and compared on ipsilateral mustard oil side with the corresponding control side (see Figure 5.3). The percentage of specific binding in the membrane fraction in mustard oil versus control and in mustard oil and drug versus mustard oil alone (Figure 5.3) were then analysed by Mann-Whitney U-Test to assess significant changes.

5.6.2 The Effect of SR 48968 on Mustard Oil-Induced PKC Translocation in Rat Spinal Cord.

Administration of SR 48968 i.v. prevented the ipsilateral mustard oil-induced translocation of [^{3}H]PDBu binding sites from cytosolic to membrane fraction (Figure 5.4). In 10 animals treated with SR 48968 the percentage of [3H]PDBu binding associated with the membrane fraction was 31.1 ± 4.2 on the mustard oil side compared to 28.6 ± 3.0 on the control (untreated) side. In animals not treated with neurokinin antagonist, corresponding values were 51.9 ± 5.9 and 26.3 ± 2.4% respectively. Thus SR 48968 prevented mustard oil-induced

Figure 5.3

The effect of unilateral mustard oil application to the skin of a distal hindlimb on the subcellular distribution of ([^{3}H]PDBu) binding sites in spinal cord.

Specific binding of [^{3}H]PDBu was determined in homogenates of hemisected spinal cord (segments L2-L5). On the control side of spinal cord (contralateral to mustard oil) this represented 12,000-15,000 dpm/assay (>85% specific) in the cytosolic fraction and 4000-5000 dpm/assay (>75% specific) in the membrane fraction. On the test side of spinal cord (ipsilateral to mustard oil application), the absolute specific binding in the membrane fraction was increased on average by 55 ± 15% (mean ± s.e.m.. n=9) and that in the cytosolic binding was only slightly reduced (<10%) from that on the control side. The proportion of specific binding recovered in the membrane fraction on the test side was significantly greater than the corresponding value on the control side ($P<0.05$, Mann-Whitney U-test).

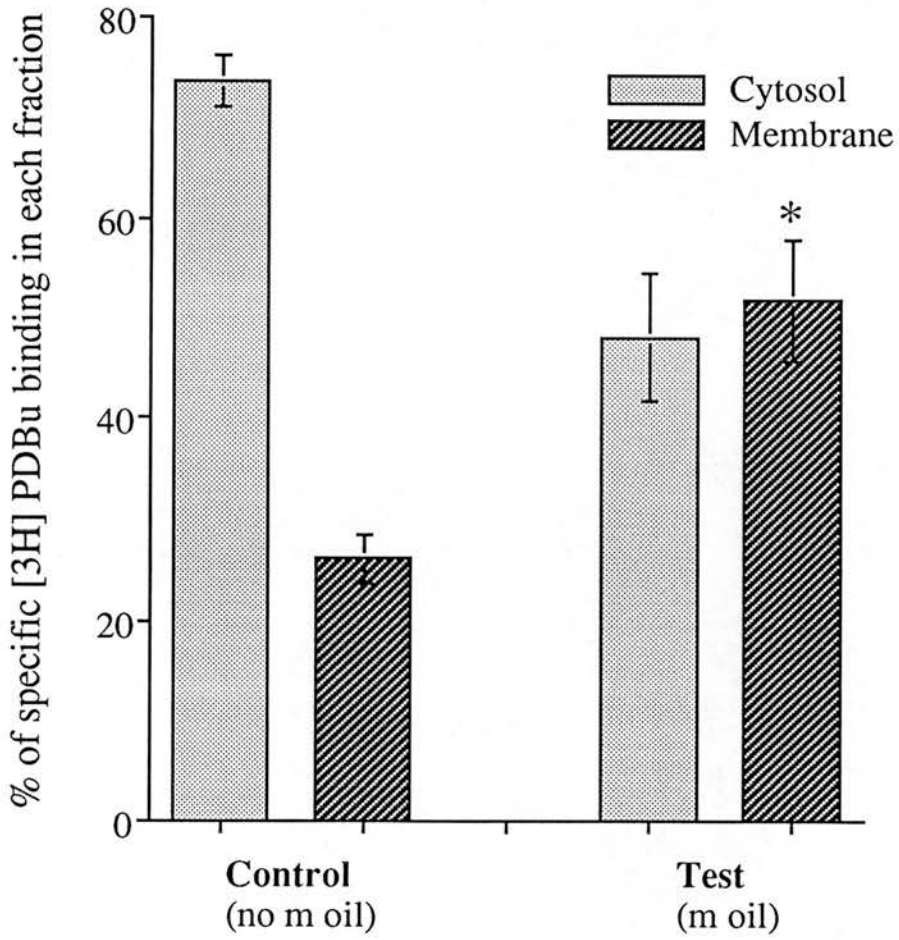
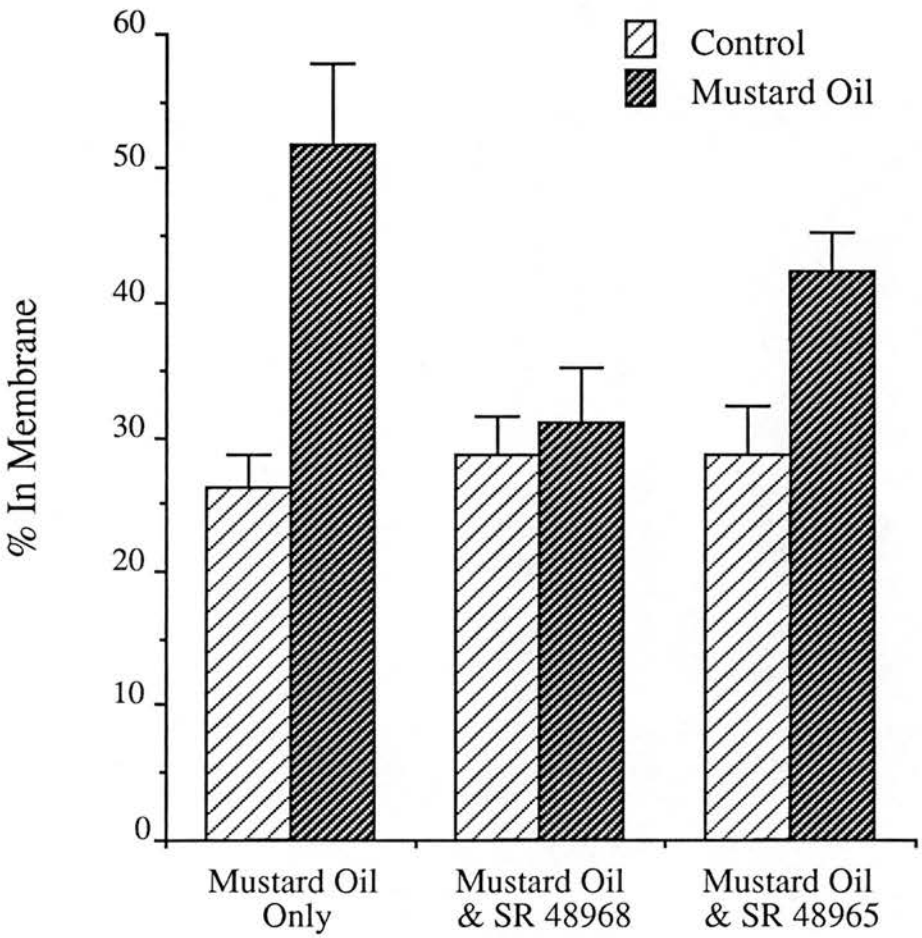

% of specific [3H] PDBu binding in each fraction
80
60
40
20
0
Cytosol
Membrane
*
Control
(no m oil)
Test
(m oil)

Figure 5.4

The effect of the NK_2 tachykinin antagonist SR 48,968 on the mustard oil-induced PKC translocation in rat spinal cord

Specific binding of [^{3}H]PDBu was determined in homogenates of hemisected spinal cord (segments L2-L5). Administration of SR 48,968 i.v. prevented the ipsilateral mustard oil-induced translocation of [^{3}H] binding associated with membrane fraction in 10 animals. The % of [3H]PDBu binding associated with membrane fraction was 31.1 ± 4.2 on the mustard oil compared to 28.6 ±3.0 on the control side. In animals not treated with neurokinin antagonist, corresponding values were 51.9 ± 5.9 and 26.3 ± 2.4% respectively.

The inactive isomer SR 48,965 failed to reproduce the suppression of [^{3}]PDBu binding site translocation seen with SR 48,968. In 9 animals the % of [3H]PDBu binding sites found in membrane fraction were 42.4 ± 2.4 and 28.7 ±3.6 on the mustard oil and control sides respectively.


60
50
40
30
20
10
0
% In Membrane
Control
Mustard Oil
Mustard Oil Only
Mustard Oil & SR 48968
Mustard Oil & SR 48965

translocation of PKC in these preparations. In one other animal, the PKC translocation was unaffected by SR 48968, in 4 other animals both the control and mustard oil-treated side showed a raised percentage of [3H]PDBu binding sites in the membrane fraction and insufficient data was obtained from another 7 animals due to technical problems. It is possible that the cases of elevated [3H]PDBu binding on both control and mustard oil-treated sides were due to an alternative drug-induced effect, but technical artefacts cannot be excluded at this stage. The inactive isomer SR 48965 failed to reproduce the marked suppression of mustard oil-induced [^{3}H]PDBu binding site translocation seen with SR 48968. In 9 animals the percentage of [3H]PDBu binding sites found in the membrane fraction were 42.4 ± 2.9 and 28.7 ±3.6 on the mustard oil treated and control sides respectively (Figure 5.4). Data from 2 further animals was unacceptable due to technical problems. Thus the mustard oil-induced translocation of PKC was only slightly attenuated by the inactive isomer SR 48965 whereas it was completely suppressed by the active NK_2 receptor antagonist SR 48968.

5.7 DISCUSSION

Two experimental models were employed to address whether protein kinase C (PKC) played any role in the sustained nociceptive responses of rat dorsal horn neurons. In the first, PKC inhibitors were ionophoretically administered whilst recording activity evoked by repeated cutaneous application of mustard oil. This *in vivo* experimental model of short-term sustained activation of C fibre nociceptive afferents has been described in Chapter 3.

All of the neurons recorded had cutaneous receptive fields on the ipsilateral hairy skin of the hindlimb, toes or foot which were multireceptive. Multireceptive neurons were always studied due to their ability to integrate both noxious and innocuous information (Iggo, 1974; Handwerker *et al*, 1975) and were identified as being located in laminae III-V using both depth measurements in combination with blue spot histology. Ejection of NaCl or vehicle were unable to reproduce any of the observed drug actions described below.

Neurons were initially located by their responsiveness to widespread manual brushing of the ipsilateral hindlimb and cutaneous fields were localised. However, only neurons which had a robust response to noxious heat were used, as these were more susceptible to showing a prolonged increase in firing rate upon application of mustard oil. Neurons were first subjected to a continuous brush-

induced activity which was maintained for a minimum period of 1 minute before ionophoresis of the highly selective PKC inhibitors was initiated and full recovery was observed before cutaneous application of the chemical algogen mustard oil. Consequent to several cutaneous applications of mustard oil (8% in paraffin oil) there was a marked increase in the background activity. Inhibitors were tested only after mustard oil-evoked activity was stable and had been continuous for at least 1-2 minutes.

From the results it can be seen that ionophoresis of each of the highly selective PKC inhibitors chelerythrine (Herbert *et al*, 1990) and GF 109203X (Toullec *et al*, 1990) profoundly and consistently reversed the mustard oil-evoked activity of dorsal horn neurons, beginning after a variable onset delay but generally within 1 minute of ionophoresis. In contrast however, GF 109203X had no discernible effect on the continuous brush evoked activity induced in a small number of multireceptive neurons.

The second experimental model employed was that of a [20-^{3}H(N)] phorbol 12,12-dibutyrate ([^{3}H]PDBu) binding assay used to assess subcellular translocation of PKC evoked in spinal cord by cutaneous application of mustard oil. PKC is activated by one of the products of inositol phospholipid metabolism, 1,2-diacylglyerol (DAG) (Nishizuka, 1989) which is mimicked by membrane-permeant, tumour-promoting phorbol esters (Castagna *et al*, 1982; Blumberg *et al*, 1984). The finding that the spinal dorsal horn contains high levels of binding sites for phorbol esters (Mantyh *et al*, 1984) and that PKC is present in the rat spinal dorsal horn (Worley *et al*, 1986; Mochly-Rosen *et al*, 1987) raised the possibility that PKC may play a functional role in sensory transmission, both in the release of putative neurotransmitters and also in signal transduction at tachykinin receptors. As we have previously demonstrated a functional role for NKA and NK_2 receptors in both acute and sustained nociceptive processing (see Chapters 2, 3 and 4) the involvement of NK_2 receptors in this process was further assessed using systemic administration of selective antagonists.

Activation of Ca^{2+}-sensitive PKC results in its intracellular redistribution (translocation) from the cytosol to the membrane (Nishizuka, 1989). Translocation/activation of PKC has been implicated in a number of sustained neuronal activated states, such those seen in long-term potentiation (e.g. Akers *et al,* 1986; Meberg *et al*, 1993), behavioural responses to formalin-induced nociception (Coderre, 1992) and the neuropathic pain after nerve injury (Mao *et al,* 1993). Therefore the biochemical experiments were carried out to examine subcellular translocation of [^{3}H]PDBu binding sites following cutaneous

application of the selective C-fibre stimulant mustard oil. Specific binding of [^{3}H]PDBu was determined in homogenates of hemisected spinal cord (segments L2-L5) and by comparing the cytosolic and membrane components from each hemisected piece of cord, it could be calculated whether PKC translocation/ activation had occurred.

The present results demonstrate that cutaneous stimulation with mustard oil leads to translocation/activation of PKC from cytosolic to membrane compartments in ipsilateral but not the contralateral (untreated, control) side of the spinal cord. However, intravenous administration of the highly selective non-peptide NK_2 antagonist SR 48968 prevented the ipsilateral mustard oil-induced translocation of [^{3}H]PDBu binding sites form the cytosolic to the membrane fraction. This is a suggestive of a selective NK_2 effect as SR 49868 has been demonstrated to be a highly potent and selective antagonist of NK_2 receptors (Advenier *et al*, 1992; Emonds-Alt *et al*, 1992,1993). Supporting this, systemic administration of the inactive (R) enantiomer of SR 48968, SR 48965 (Emonds-Alt *et al*, 1992) failed to reproduce the marked suppression of mustard oil-induced [^{3}H]PDBu binding site translocation seen with SR 48968.

Evidence has been provided consistent with a role of PKC in central sensitisation. Behavioural nociceptive responses to subcutaneous formalin injection are enhanced by activators of PKC and PKC inhibitors (Coderre, 1992), whereas low affinity blockers of phospholipase A_2 and of protein kinase A (PKA) were ineffective. In the presence of a permeabilising agent, a PKC pseudosubstrate inhibitory peptide was also effective (Coderre and Yashpal, 1994), although none of the agents modified acute thermal or mechanical flexion reflexes in normal animals. Moreover, infusion of a PKC activator (the phorbol ester TPA) into the dorsal horn facilitates neuronal responses to NMDA in the dorsal horn *in vitro* (Gerber *et al*, 1989) and produces an increase in the background activity of spinothalamic tract (STT) neurons and a delayed facilitation of neuronal non-nociceptive mechanical responses *in vivo* (Palecek *et al*, 1994). When a phorbol ester which does not activate PKC (α-TPA) was applied, no significant changes in background or evoked activity of STT cells were observed providing evidence that PKC may play an important role in the process of sensitisation of dorsal horn neurons to innocuous stimuli.

Use of the models of peripheral mononeuropathy and monoarthritis has further implicated the involvement of PKC in long-term central changes elicited by inflammation (Hayes *et al*, 1992; Mao *et al*, 1993; Tolle *et al*, 1994). Both the pattern of increased membrane bound PKC and behavioural nociceptive responses

associated with the loose ligation neuropathy model were reliably reduced following administration of PKC inhibitors (Hayes *et al*, 1992; Mao *et al*, 1993). Likewise, Tolle *et al*, (1994) observed increased [^{3}H]PDBu binding coinciding with monoarthritis-induced inflammation. Moreover, evidence suggests that PKC has a critical role in the mediation of another model of synaptic enhancement, that of LTP (Akers *et al,* 1986; Reymann *et al,* 1988; Malinow *et al,* 1989; Huang *et al,* 1992; Wang and Feng, 1992; Meberg *et al,* 1993). Interestingly, the increased expression of preprodynorphin mRNA elicited in ipsilateral superficial dorsal horn by intraplantar carrageenan (like mustard oil-induced activation here (see Chapter 3), is blocked by NK_2 receptor antagonists (Parker *et al*, 1993), again consistent with a role of a signal resulting from phosphoinositide hydrolysis such as PKC activation.

This study demonstrated a role for NK_2 receptor agonists in the PKC-induced sustained neuronal activation following cutaneous application of mustard oil. However, other mediators are likely to contribute additionally to mustard oil-induced PKC activation and sensitisation of dorsal horn neurons, for example NK_1, and metabotropic glutamate receptors have both been shown to be involved in the mustard oil-induced sustained activation of C fibres (see Chapter 4 and Young *et al,* 1994) and are therefore likely to contribute to similar subcellular transduction mechanisms. The NK receptors and probably the metabotropic glutamate receptor subtype involved in sensitisation, act through phosphoinositide hydrolysis, calcium mobilisation and stimulation of protein kinase C (PKC) (Hanley *et al,* 1980; Bristow *et al,* 1987; Thomson *et al,* 1994).

In agreement, various other studies have indicated a role for tachykinins as well as metabotropic glutamate receptors in the PKC-induced sensitisation. In dorsal horn slices *in vitro*, activation of PKC by phorbol esters facilitated neuronal depolarising responses to NMDA (in the presence of tetrodotoxin) and also increased the release of excitatory (and other) amino acids evoked by dorsal root stimulation (Gerber *et al*, 1989). Moreover, the potentiation of NMDA currents produced by neurokinins in isolated dorsal horn neurons can be inhibited by the non-selective kinase inhibitor staurosporine; mimicked by the PKC activator PDBu and (although PKA activation is not likely to be a prominent direct consequence of SP action) can also be mimicked by activators of PKA (Gerber *et al*, 1989; Rusin *et al*, 1992; Cerne *et al*, 1993). However, in the neonatal spinal cord/tail preparation, staurosporine blocked the NK_2 but not the NK_1 agonist-induced VRP and the neurokinin A enhancement of excitatory amino acid response (Urban *et al*, 1994) suggesting that NK_2 but not NK_1 receptor-induced

depolarisation involves activation of PKC. This is surprising as both NK_1 and NK_2 receptors are linked to the phosphoinositide pathway, although it is possible that they influence other second messenger systems to somewhat different extents. Nevertheless, this is not in agreement with the results obtained from Coderre and Yashpal (1994). In this study, the enhancement of formalin-induced nociceptive behaviour elicited by glutamate or SP, was also reversed by pre-treatment with a highly selective PKC inhibitor, PKC(19-26), (Coderre and Yashpal, 1994). The analgesic effects of this PKC inhibitor were restricted to the tonic intermediate and late phases of the formalin test and did not affect the acute early phase. In agreement with our results, this suggests that the intracellular events triggered by these second messengers are critically involved in mediating the plasticity or central sensitisation that leads to persistent nociception. Recent experiments have shown that the facilitation of dorsal horn activity elicited by metabotropic glutamate agonist is similarly blocked by selective PKC inhibitors (Young *et al*, unpublished observations).

A molecular mechanism whereby PKC activation could act to promote the function of NMDA receptor channels has been described, where receptor phosphorylation results in diminished Mg^{2+} block and increased conductance at a given membrane potential (Chen *et al*, 1992; Tingley *et al*, 1993). Clearly either neurokinin receptors or phosphoinositide-hydrolysing metabotropic glutamate receptors could initiate such a mechanism, perhaps underpinning the synergistic contribution they make, with NMDA receptors, to dorsal horn sensitisation. Consistent with this body of evidence for a role of PKC in sensitisation, is a recent report that the ongoing basal levels of inositol 1,4,5-triphosphate are elevated in the spinal cord ipsilateral to intraplantar injection of Freud's adjuvant (Igwe and Ning, 1994).

Interestingly PKC may also play a role in the regulation of tedrodotoxin-insensitive Na^{+} channels (Thio and Sontheimer, 1993) and Ca^{2+}channels (McEwan and Mitchell, 1991). Recent evidence suggests that noxious stimulation may produce an increase in intracellular Ca^{2+} in spinal cord dorsal horn neurons that influences nociceptive excitability (Coderre and Melzack, 1992). Neurotransmitters released in response to noxious stimulation are known to affect the intracellular levels of Ca^{2+}. Whereas glutamate and aspartate stimulate the influx of Ca^{2+} through NMDA receptor operated channels (MacDermott *et al*, 1986), SP produces an elevation in intracellular Ca^{2+} by altering its release from intracellular stores (Womack *et al*, 1988) as well as increase in Ca^{2+} influx through voltage-gated Ca^{2+} channels (Womack *et al*, 1989). L-type Ca^{2+}

channel antagonists inhibit 'wind-up' in turtle spinal cord slices *in vitro* and are analgesic in the formalin test of behavioural nociception upon systemic but not local administration (Russo and Hounsgaard, 1994; Miranda *et al*, 1992). Low doses of L-channel blockers applied intrathecally are reported to synergise with the antinociceptive effects of morphine in the Tail-flick test: N and P-type channel blockers applied by intrathecal infusion inhibited not only the late phase of formalin nociceptive behaviour, corresponding to central sensitisation, but also initial short-latency activity and displayed some motor side effects (Malmberg and Yaksh, 1994).

In conclusion, under the present circumstances at least, PKC is strongly implicated as a crucial mediator of the activation of dorsal horn neurons evoked by a sustained nociceptive challenge. It is clear that PKC activation can result from NK_2 neurokinin receptor mechanisms but likely that both NK_1 and metabotropic glutamate receptors also contribute to inflammatory central sensitisation and hyperalgesia. Therefore blockade of a common intracellular transducer represents a very important target for new analgesics and may be a much more effective route than attempting to design a cocktail of receptor antagonists.

CHAPTER 6:
Mustard Oil-Induced c-fos mRNA Expression In Rat Spinal Cord.

6.1 AIMS

The aim of these experiments was to use the technique of *in situ* hybridisation histochemsitry to examine the expression of c-fos mRNA within the rat spinal cord. Using cutaneous application of mustard oil as a relatively short-term model of sustained nociception accompanied by central sensitisation, the facilitated expression of c-fos was quantified throughout the superficial laminae of the spinal cord. Control studies were then carried out using peripheral application of paraffin oil to determine the constitutive and facilitated areas of c-fos expression. The exclusive mustard-oil facilitated expression of c-fos could now be established as a marker and used to investigate the effects of intravenous administration of non-peptide NK_1 and NK_2 tachykinin antagonists on the spinal changes accompanying cutaneous application of mustard oil.

6.2 MATERIALS

Animals: Male Wistar rats were obtained from Charles River UK Ltd, Margate, Kent, UK.

Anaesthetics: Alpha-chloralose and urethane were obtained from Sigma Chemical Company, Poole, Dorset, UK; Halothane (flurothane) was obtained from ICI Pharmaceuticals, Macclesfield, Cheshire, UK.

Molecular Reagents: All laboratory chemicals used were of molecular biology grade and dry compounds were weighed onto weighboats that had been cleaned with ethanol or sterile filter paper. Equipment and solutions were sterilised by autoclaving at 120°C (10 lb/ins pressure) for 1 hour. The reagents used were as follows: sodium chloride (NaCl), ammonium acetate, poly-L-lysine, diethyl pyrocarbonate (DEP), dextran sulphate, Haematoxylin, Trizma Hydrochloride, Trizma Base, yeast tRNA, salmon sperm DNA (sonicated), Bovine albumin (fatty acid free) (BSA), sodium citrate and dithiothreitol (DTT) were all obtained from Sigma Chemical Company, Poole, Dorset, UK: Allyl-isothiocyanate (mustard oil) was purchased from Aldrich Chemical Company Ltd., Gillingham, Dorset, UK; Paraffin was purchased from Scotlab, Lanarkshire, UK; Terminal

deoxynucleotidyl transferase and potassium cacodylate tailing buffer were obtained from Gibco BRL, Paisley, UK; Phenisol developer, fixer and K5 emulsion were obtained from Ilford Scientific Product, Cheshire, UK: Isopentane, formamide, di-sodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, paraformaldehyde, Depex mounting medium, potassium dichromate, silica gel, sulphuric acid, Haematoxylin and Eosin were obtained from BDH Chemicals Ltd, Poole, Dorset, UK; glycogen and ribonuclease A were obtained from Boehringer Mannheim, Lewes, East Sussex, UK.

Oligomer Probe: A 48-base oligodeoxyribonucleotide was synthesized and HPLC-purified by Oswel Chemicals, Edinburgh University Chemistry Department.

Radiolabelled Compound: Deoxyadenosine 5'-[α ^{35}S] triphosphate (with specific activity > 1 x 10^3 Ci/mmol) was obtained from New England Nuclear Research Products, Du Pont de Nemours, Dreiech, Germany.

Drugs: RP 67580 was a gift from Dr C Garret, Rhone-Poulenc Recherché-Development, France and SR 48968 was a gift from Dr X Emonds-Alt, Sanofi Recherché, France.

6.3 METHODS

6.3.1 Animals

Adult male Wistar rats (240-320g) were used as subjects. Animals were housed in groups of 3 or 4 in standard plastic cages, maintained on 12 hour light: 12 hour dark cycle at 22-23°C; food and water were freely available throughout experiments.

6.3.2 Treatment and Tissue Preparation

(See Figure 6.1 for a Summary Diagram)

(a) Anaesthetised Paraffin Oil Control.

Rats were briefly anaesthetised with halothane and both hind limbs and back were shaved, the jugular vein was cannulated and α-chloralose/urethane was administered as described in Chapter 2. The left hindfoot was painted with paraffin oil 3 times over 10 minutes and covered up as described in Chapter 3. After 45 minutes, the rat was killed by cervical dislocation following stunning and

Figure 6.1

Summary diagram showing the preparation of spinal cord for *in situ* hybridisation histochemistry

(a) Tissue Preparation- Adult male rats were anaesthetised with halothane, both legs were shaved and intravenous cannula inserted for administration of anaesthetic or antagonists. Over a period of 15 minutes, mustard oil (8%) or paraffin oil was painted three times over the hindlimb and paw. After 30 minutes, the spinal cord was removed and rapidly frozen in isopentane at -45°C for 3 minutes.

(b) Hybridisation- 10μm thick fresh-frozen segments of intact spinal cord were cut on a cryostat and thaw mounted onto subbed slides. The sections were prepared for *in situ* hybridisation histochemistry, as outlined.

In Situ Hybridisation Histochemistry

A: Tissue Preparation

Adult male rats.
↓
Anaesthetised with halothane
↓
Both legs shaved
↓
Intravenous cannula inserted
↓
8% mustard oil or paraffin oil, painted 3X over hindlimb and paw.
↓
After 30 mins, spinal cord quickly removed, frozen at -45°C in isopentane for 3 mins.
↓
Stored at -70°C until required.
↓
10μm thick sections cut on the cryostat, thaw mounted on 'subbed' slides.
↓
Slides stored at -70°C until required.

B: Hybridisation

Tissue sections thawed.
↓
Fixed in 4% paraformaldehyde-10 mins washed 2X in 0.1M PBS.
↓
Acetylated to reduce non-specific binding.
↓
Dehydrated in ethanol.
↓
Hybridisation with [^{35}S]-labelled c-fos oligonucleotide overnight at 37°C, the hybridisation buffer containing 1 x 10^6 DPM/slide and 50% formamide.
↓
Post-hybridisation washes at 37°C.
2X SSC - 2hrs
1X SSC - 2hrs
0.5X SSC - 2hrs
↓
Dehydration through increasing steps of 50% to 90% buffered ethanol.
↓
Air-dried overnight.
↓
Dipped in Ilford K5 emulsion.
↓
Exposed at 4°C for 35 days in a light-proof box.
↓
Developed and fixed.
↓
Stained in haematoxylin and eosin.
↓
Sections examined for positive cells by light microscope.
↓
Silver grain density carried out using an Optilab Image Analysis System

the spinal cord was removed as rapidly as possible, usually 4-5 minutes after performing a laminectomy (using sterile (boiled) dissection instruments) to reveal sections L1-L6 of the spinal cord. The dura was carefully removed with fine forceps and the piece of spinal cord was transversely cut to produce two equal sized pieces. Each piece was carefully dissected free, dorsal roots removed and positioned vertically onto a drop of Bright Cryo-M-Bed Embedding Compound on an autoclaved metal chuck. The free end of the tissue was held lightly with forceps and the chuck was gently lowered and rapidly frozen for 3 minutes in a container of isopentane previously cooled to -45 to -50°C on 'dry-ice' bath (solid carbon dioxide). Care was taken that the temperature was in the correct range to prevent the tissue from cracking. Each piece of lumbar cord was then wrapped in cling-film and stored in a sealed sterile pot at -70°C until it was sectioned.

(b) Conscious Paraffin Oil Control.

Experiments were carried out to determine if the anaesthetic used (Halothane) had any effect on the level of constitutive c-fos expression. Thus experiments were carried out in an identical manner to those of the anaesthetised rats. Under halothane anaesthesia, the rats' legs were shaved, paraffin oil was painted onto the leg three times over 15 minutes before it was bandaged up with plastic film and sticky tape. However, instead of maintaining anaesthesia for a further 30 minutes as in the protocol described above, the rat was now put into a wire mesh restraining cage, where it regained consciousness for a further 30 minutes. It was then killed and the tissue was processed as described above.

(c) Mustard oil Only

Rats were briefly anaesthetised with halothane and both hind limbs and the back were shaved. The jugular vein was cannulated and then the anaesthetic was changed from halothane to an α-chloralose / urethane mixture (35mg/kg α-chloralose and 700mg/kg urethane). This was administered slowly until a regular, steady respiratory rate was achieved. Anaesthetic levels were continuously monitored by rate of respiration and corneal reflex; 0.1ml aliquots of anaesthetic were administered as required. Three applications of 8% mustard oil were painted onto the left hindfoot and leg over a 10 minute period. The treated leg was then carefully taped up to prevent cross-contamination of mustard oil to the contralateral hindlimb.

After 45 minutes the rats were killed by stunning and cervical dislocation and the tissue was processed as described above.

(d) Mustard Oil and Non-Peptide Antagonists or Vehicle

Rats were briefly anaesthetised with halothane, both hind-limbs shaved, jugular vein cannulated and α-chloralose/urethane administered as before. Once a stable anaesthetised state was maintained, the antagonist or vehicle was given as an intravenous injection into the jugular vein. The left hindfoot and leg were painted with mustard oil 3 times over 10 minutes and then taped up as before. For SR 48968, SR 48965 and vehicle, only one dose was given, however RP 67580 and RP 68651 were given every 15 minutes, due to their shorter half-life (Laird *et al,* 1993). After 45 minutes, the spinal cord was removed and tissue frozen as described above.

6.3.3 Subbing Slides

(See Figure 6.2 for a Summary Diagram)

Glass microscope slides were first cleaned by immersion in a chromic acid bath for 24 hours. The slides were thoroughly washed to remove all traces of acid before washing in 2% Decon for 3 hours followed by H_2O and then diethyl pyrocarbonate (DEP) H_2O (5-6 drops of DEP in 1 Litre of distilled H_2O). The slides were then soaked in absolute ethanol for 10-15 minutes to remove any remaining grease. Once dry, the slides were ready for coating. This process involves firstly, dipping the slides in 0.2M HCl, followed by DEP H_2O and acetone for 3 minutes each before baking in an oven at 50-60°C for 15 minutes to remove any final RNases. The racks of slides were then dipped in poly-L-lysine solution (Sigma), twice for 10 seconds each time and then rinsed in DEP H_2O for 10 minutes to decrease static and dust attraction. After drying in the oven overnight at 50-60°C the slides were boxed, sealed tightly and used within 5 to 6 weeks.

6.3.4 Cutting Sections

An OTF/AS Bright cryostat (Bright Instrument Company Ltd., Cambridgeshire, UK) was used for sectioning of the rat spinal cord. The temperature was set to between -18°C and -25°C depending on the tissue mass of the specimen. A disposable blade was first rinsed in xylene to remove the lubricant, followed by ethanol to clean and remove any excess grease before being set at an angle of 5° to the mounted chuck.

The chuck was transported from the freezer to the cryostat on dry ice to ensure that the -70°C temperature was maintained. It was secured in the cryostat and left for 1-2 hours to equilibrate to the set temperature.

Figure 6.2

Summary diagram showing the protocol used for subbing slides

Glass microscope slides were first cleaned with chromic acid and rinsed in copious amounts of fresh water before being air-dried overnight. Slides were then ready to be subbed as outlined.

Subbing Slides

Cleaning Slides

Glass microscope Slides.

↓

Cleaned in chromic Acid- 24hrs.

↓

Rinse in H_2O overnight.

↓

Wash 3 hrs in 2% Decon.

↓

Wash in H_2O, 1 Day.

↓

2 x 15mins, Dep H_2O.

↓

Absolute ethanol 10-15 mins.

↓

Air-Dried.

Subbing Slides

↓

0.2N NaCl, 3 mins.

↓

Dep H_2O, 3 mins.

↓

Acetone, 3 mins.

↓

Dried, 50-60°C, 15 mins.

↓

2 x 10 sec Poly-L-Lysine.

↓

Dep H_2O, 10 mins.

↓

Dried, 50-60°C, overnight.

↓

Slides boxed and stored with dessicant.

The cryostat was initially set to cut 30μm sections and the tissue was trimmed until a full transverse section of the cord was clearly visible when stained with methyl blue and viewed at x40 under a light microscope. To minimise damage to the dorsal horns, which have little surrounding white matter to protect them during the cutting, the chuck was rotated so that both dorsal horns were the last part of the cord to be sectioned by the blade. A marker hole was made in the right ventral horn using a sterile needle, prior to cutting.

The setting was then changed to cut 10μm thick sections and 10 alternate sections were thaw mounted per poly-L-lysine subbed slide. One section was taken every 5 sections for histology, stained with methyl blue and viewed under the light microscope to ensure that the sections had no cracks or edges missing or were folded. Gloves were always used and the blade was frequently cleaned with ethanol to minimise RNAse contamination.

The mounted sections were stored in the cryostat until cutting was finished, then they were transferred on dry ice to the -70°C freezer, where they were stored in labelled Kartell boxes with silica gel and sealed with electrical tape to prevent frost accumulating on the slides.

6.3.5 Probe Labelling

A 48-base oligodeoxyribonucleotide was synthesized and HPLC-purified by Oswel Chemicals, Edinburgh University Chemistry Department. The c-fos oligomer was complementary to the base sequence 582-630 of the c-fos mRNA (Curran *et al,* 1987), (see Figure 6.3). The probe is designed against the base sequence of the conserved DNA binding domain in Fos-family proteins, therefore it should be noted that it will not necessarily display specifically c-fos, but also closely related proteins.

The method for labelling the oligonucleotide involves addition of [^{35}S] dATP to the 3' end of the oligonucleotide with the enzyme terminal deoxytidyl transferase (TdT), followed by extraction of the labelled probe. The following protocol was followed:-

The purified probe was stored at -70°C, thawed and diluted by 2μl probe : 5.3μl dH_2O (14 OD/ml). 2μl of this dilution was taken to give 25pmol probe. To label the probe, the following were added to a 1.5ml eppendorff tube and heated in a 32°C waterbath for 15 minutes: 2μl (25pmol) probe; 10μl tailing buffer; 38μl ddH_2O (autoclaved distilled water); 5μl [^{35}S]dATP (>1000Ci/mmol, New England Nuclear, USA). Once the reaction had reached equilibrium (after 15 mins), 5μl TdT enzyme (Gibco BRL), was added.

Figure 6.3

Nucleotide Sequence of c-fos (rat)

The nucleotide sequence proceeds in the 5' to 3' direction. The deduced amino acid sequence from the open reading frame is shown below the nucleotide sequence. The c-fos probe used was complemetary to bases encoding amino acids 582-629 and is highlighted in a box above the nucleotide sequence.

(From Curran *et al,* 1987)

CGCAACTGAGAAGACTGGATAGAGCCGGCGGAGCCGCGAACGAGCAGTGACCGCGCTCCCACCCAGCTCTGCTCTGCAGCTCCCACCAG

TGTCTACCCCTGGACCCCTCGCCGAGCTTTGCCCAAACCACGACCATGATGTTCTCGGGTTTCAACGCGGACTACGAGGCGTCATCCTCC

M M F S G F N A D Y E A S S S

CGCTGCAGTAGCGCCTCCCCGGCCGGGGACAGCCTTTCCTACTACCATTCCCCAGCCGACTCCTTCTCCAGCATGGGCTCCCCTGTCAAC

R C S S A S P A G D S L S Y Y H S P A D S F S S M G S P V N

ACACAGGACTTTTGCGCAGATCTGTCCGTCTCTAGTGCCAACTTTATCCCCACGGTGACAGCCATCTCCACCAGCCCAGACCTGCAGTGG

T..Q..D..F..C..A..D..L..S..V..S..S..A..N..F..I..P..T..V..T..A..I..S..T..S..P..D..L..Q..W..

CTGGTGCAGCCCACTCTGGTCTCCTCCGTGGCCCCATCGCAGACCAGAGCGCCCCATCCTTACGGACTCCCCACCCCGTCGACCGGGGCT

L V Q P T L V S S V A P S Q T R A P H P Y G L P T P S T G A

TACGCCAGAGCGGGAGTGGTGAAGACCATGTCAGGCGGCAGAGCGCAGAGCATCGGCAGAAGGGGCAAAGTAGAGCAGCTATCTCCTGAA

Y A R A G V V K T M S G G R A Q S I G R R G K V E Q L S P E

CGACGTCGCTTCACGGCCTTAGCCTCCTCCCTCGACTGTCTATGCGAG

GAGGAAGAGAAACGGAGAATCCGAAGGGAAAGGAATAAGATG**GCTGCAGCCAAGTGCCGGAATCGGAGGAGGGAGCTGACAGATACGCTC**

E..E..E..K..R..R..I..R..R..E..R..N..K..M..A..A..A..K..C..R..N..R..R..R..E..L..T..D..T..L..

CAAGCGGAGACAGATCAACTTGAAGACGAGAAGTCTGCGTTGCAGACCGAGATTGCCAATCTACTGAAAGAGAAGGAAAAACTGGAGTTT

Q A E T D Q L E D E K S A L Q T E I A N L L K E K E K L E F

ATTTTGGCAGCCCACCGACCTGCCTGCAAGATCCCCAATGACCTGGGCTTCCCAGAGGAGATGTCTGTGACCTCCCTGGACTTGACTGGG

I L A A H R P A C K I P N D L G F P E E M S V T S L D L T G

GGTCTGCCTGAGGCTACCACCCCAGAGTCTGAGGAGGCCTTCACCCTGCCTCTTCTCAATGACCCTGAGCCCAAGCCATCCTTGGAGCCG

G L P E A T T P E S E E A F T L P L L N D P E P K P S L E P

GTCAAGAACATTAGCAACATGGAGCTGAAGGCTGAACCCTTTGATGACTTCTTGTTTCCGGCATCATCTAGGCCCAGTGGCTCGGAGACT

V K N I S N M E L K A E P F D D F L F P A S S R P S G S E T

GCCCGCTCTGTGCCAGATGTGGACCTGTCTGGTTCCTTCTATGCAGCAGACTGGGAGCCTCTGCACAGCAGTTCCCTGGGGATGGGGCCC

A R S V P D V D L S G S F Y A A D W E P L H S S S L G M G P

ATGGTCACAGAGCTGGAGCCCCTGTGCACTCCCGTTGTCACCTGCACTCCCAGCTGCACTACCTATACGTCTTCCTTTGTCTTCACCTAC

M V T E L E P L C T P V V T C T P S C T T Y T S S F V R T Y

CCCGAGGCTGACTCCTTCCCTAGCTGCGCAGCTGCCCACCGAAAGGGCAGCAGCAGCAACGAGCCCTCCTCTGACTCACTGAGCTCGCCC

P E A D S F P S C A A A H R K G S S S N E P S S D S L S S P

ACACTGCTAGCCCTGTGAGCAGTCAGAGAAGGCAGGGCAGCCGGCACTGACTGAGCTGGTGCATTACAGAGAGGAGAAACACGTCTTCCC

T L L A L

TCGAGGGGTTCCCGTAGACCTAGGGAGGACCTTATCTGTGCGTGAAACACACCAAGCTGTGGACCTCAAGGACTTGAAAGCATCCACATC

TGGACTCCAGTCCTCACCTCTTCCGCAGATGTAGCAAAAAAACAAAAAAACAAAACAAAAAAAAAACAAAACAAAAAATCAAAAGCAACC

GCATGGAGTGTATTGTTTGTAGTGACACCTGAGAGCTGGTAGTTAGTAGCATGTGAGCCAGGCCTGGGTCTGTGTCTCTTTTCTCTTTCT

CCTTAGTCTTCTCATAGCATTAACTAATCTGTTGGGTTCATTATTGGAATTAACCTGGTGCTGGATATTTTTCGGATTGTATCTAGTGCA

GCTGATTTTAACAATACCTACTGTGTTCCTGGCAATAGTGTGTTCCAATTTAGAAATGACCAATATTAAACTAAGAAAAGATAGAACTTT

ATTTTCCGGTAGATAGAAATAAATCGCTATATCCACGTACTGTAGCTCTTCAGCGTCCATGTTCATTGTCATGTAACTGATCATGCATTG

TTGAGGTGGTCTGAATGTTCTGACATTAACAGTTTTCCATGAAAACGTTTTATTGTGTTTTCAATTTATTTATTAAGATGGATTCTCAGA

TATTTATATTTTTATTTTATTTTTTTCTATCCTGAGGTCTTTCGACATGTGGAAAGTGAATTTGAATGAAAAAATTTTAAGCATTGTTTG

CTTATTGTTCCAAGACATTGTCAATAAAAGCATTTAAGTTGAATGCGAAAAAAAAAAAAAAAAAAAA

The reaction was stopped by placing on ice for 15 minutes and the sample was then purified through a Nu-Clean D25 Disposable Spun Column (IBI). 1µl of pre- and post- spun samples were pipetted onto small paper squares in plastic scintillation tubes. 4ml of scintillation fluid was added to each tube and it was vigorously shaken. Each tube was counted for β radioactivity on a scintillation counter. A successful labelling reaction gave a probe specific activity of 1-2 x 10^3 Ci/mmol and a labelling efficiency of >65%. This labelled oligonucleotide was either used immediately or stored at -20°C overnight.

6.3.6 Hybridisation

(See Figure 6.1 for Summary diagram).

The slides, each holding 10 sections were removed from the -70°C freezer, put in racks and brought to room temperature for a minimum period of 2 hours. The tissue was first fixed in 4% paraformaldehyde in 0.1M PBS, washed twice in 0.1M PBS, acetylated to reduce non-specific binding and then dehydrated through increasing concentrations of buffered alcohol (50,70,80,90 and 100% for 2 minutes each).

Hybridisation was carried out under conditions similar to those described by Young *et al,* (1986). It was carried out in plastic containers containing foam saturated with the following soaking solution (10-15ml per box of 4* standard sodium citrate (0.03M NaCl and 3mM Tri Sodium Citrate) (4* SSC): de-ionised formamide. Four slides were placed in each container and 20µl of hybridisation buffer was carefully dropped onto each section, taking care not to damage the section. There was 5 x 10^3dpm of probe per µl of hybridisation buffer containing 10% (wt/vol.) dextran sulphate, NaCl (600mM, vol:vol), bovine serum albumin (0.1%, vol:vol), salmon sperm DNA (0.1%, wt:vol), glycogen (0.005%, wt:vol), yeast tRNA (0.002%, wt:vol), formamide (50%, vol:vol), dithiothreitol (10µl, 1M per ml). Each container was sealed and placed in an incubator at 37°C for 20 hours.

After this time post hybridisation washes were carried out over 6 hours at 20°C below the calculated melting temperature for the probe, in decreasing concentrations of standard sodium citrate (SSC) solution from 30mM NaCl plus 3mM trisodium citrate, to 7.5mM NaCl plus 0.75 mM trisodium citrate. Slides were dehydrated through steps of 50, 70 and 90% buffered ethanol and air dried overnight.

6.3.7 Apposing Slides

Ilford K5 emulsion was melted in a waterbath in the darkroom at 45°C for approximately 45 minutes before being diluted 1:2 (vol:vol) with distilled water. The slides were dipped in emulsion and air-dried overnight before being boxed with silica gel and sealed with electrical tape, aluminium foil and black photographic plastic to eliminate any light.

6.3.8 Developing and Staining Slides

Exposure took place at 6°C for 4-5 weeks. After this time, the slides were brought to room temperature for >2 hours before being developed in Ilford Phenisol (diluted 1:4 (vol:vol) with distilled water) in a darkroom. This took 4 minutes with gentle agitation followed by a fixing process in Ilford Hypam Fixer (diluted 1:4 (vol:vol) with distilled water) for 4 minutes. The slides were then rinsed for five 10 minute periods in copious amounts of fresh distilled water before being stained.

The slides were then lightly stained with Mayers Haematoxylin (2.5 minutes), washed and then stained with 1% Eosin in 80% alcoholic solution (3 minutes) followed by dehydration through 95% and 100% ethanol (2 minutes each step). Finally, lipid was extracted in Histoclear (4 minutes) before being mounted under Depex mounting medium.

6.3.9 Analysis

(a) Primary Examination Under the Light Microscope

Initially each slide was examined carefully by light microscope under x40 magnification to enable a rough estimation of c-fos mRNA to be carried out. Both sides of the spinal cord were scrutinised on each section and the level of mRNA expression assessed in both lateral and medial superficial dorsal horn (Laminae I-II) and additionally, deep dorsal horn (Laminae IV-V). This was achieved by visually grading the level of mRNA expression from 0-5, with 0 representing no expression and 5 that of maximum expression, i.e. very dense clustering of black silver grains around each nucleus.

A diagrammatic 'map' was prepared for each spinal cord section which detailed the position of labelled cells and acted as a reference map when using image analysis.

(b) ***Image Analysis***

An image analysis system was used to evaluate each slide. This consisted of a Zeiss Axioskop 20 microscope with a CCD video camera attached which relayed the image from the microscope to an Apple Macintosh M1298 computer.

The slide under examination was first cleaned with alcohol and then viewed at x40 under the light microscope. The appropriate section was selected and the first field was set, this was always at the medial edge of the dorsal horn. The microscope light was then set to a level for optimum cell and silver grain display.

The image 144 VDM-F programme was loaded onto the computer and a 'macro' (a pre-determined set of commands that are triggered by the press of a selected key on the computer panel) was set up to open the 'Excel' programme within this application, calibrate the image for x40 magnification and to analyse the labelled particles within the manually set area desired. This was achieved by first setting the camera 'live' which opens the camera and transmits a live image to the computer screen. The image was completely defocused to set a 'blank field' which was consequently subtracted from each image thus minimising any unwanted background markation.

A second macro was set up to enhance the image and thus make determination of silver grain density more accurate. This consisted of sharpening the image, enhancing the contrast followed by a second sharpen. The filed data was then ready for determination of silver grain density. The three densest (blackest) areas of silver grain were chosen in each field and then marked onto the section map. Using the computer mouse a line was drawn around the first region of interest (ROI).

A third macro was set up to measure this area and to set the density slice which was then adjusted manually to highlight only the silver grains and not any background staining. The number of silver grains per μm^2 were analysed by the computer and the values were pasted into the appropriate position on the spreadsheet.

The ROI area was then moved to an adjacent area of background which always included an unlabelled nucleus and analysis was carried out as before taking care not to move the set point of the density slice. The value was pasted into the appropriate column on the spreadsheet such that all the values were now 'background adjusted'.

6.4 RESULTS

6.4.1 Detection of c-fos mRNA With In Situ Hybridisation Histochemistry-Confirmation of Technique.

The c-fos mRNA expression detected in both the mustard oil and paraffin oil treated animals was found in the same general areas of the dorsal horn observed by other investigators after peripheral nociceptive stimulation (Hunt *et al,* 1987; Abbadie and Besson, 1993)

6.4.2 Control Studies

(a) RNAse Controls

RNAse controls were undertaken to ensure that no non-specific labelling of cells was present when using the In Situ Hybridisation Histochemistry (ISHH) technique. The results showed that after RNAse treatment, no specific labelling of c-fos cells was detected, however a low level of non-specific background activity could be seen.

(b) Paraffin Oil Controls

The effects of unilaterally painting paraffin oil three times onto the receptive field with a soft paintbrush over 15 minutes was carefully examined in anaesthetised rats. The levels of c-fos mRNA expression in the superficial layers (laminae I-II) after unilateral application of paraffin oil to the left hindlimb, were examined by ISHH, a typical example of this can be seen in Figure 6.4. There was a low and even distribution of silver grains spread through the spinal cord (both treated and untreated sides) with no apparent difference between medial and lateral areas of the superficial layers.

The table in Figure 6.5 demonstrates the mean silver grain density (μm^2) of the superficial layers of the dorsal horn following unilateral paraffin oil application. This was a mean of 135 regions of interest taken from 3 rats. From the histogram in Figure 6.6, it can be seen that there is no significant difference between the experimental (paraffin oil treated) side and the control (untreated) side of the spinal cord, ($P>0.05$, Matched Pair Students t-test).

The distribution of c-fos mRNA in the deeper layers of the spinal cord (Laminae IV-V), although not examined in such great detail, appeared to mirror the expression pattern of the superficial layers, with no difference between the 'paraffin oil' and 'mustard oil' sides. Thirty five sections, taken from four rats

Figure 6.4

c-fos mRNA expression in the rat lumbar spinal cord following cutaneous paraffin oil application.

Low power lightfield photographs showing the typical levels of c-fos mRNA expression over the superficial dorsal horn following cutaneous application of paraffin oil, as identified by ISHH. Examples of positively-labelled neurons, characterised by a dense aggregation of silver grains around haematoxylin-stained nuclei, are represented by filled arrows. The open arrows demonstrate examples of non-expressing nuclei with low and evenly distributed background density of silver grains. **A** Demonstrates the basal expression, in the side contralateral to the unilateral application of paraffin oil. Photographs were taken of overlapping areas and then aligned to show the full mediolateral extent of the superficial region the dorsal horn. **B** Demonstrates the expression obtained in the opposite dorsal horn, ipsilateral to unilateral application of paraffin oil.

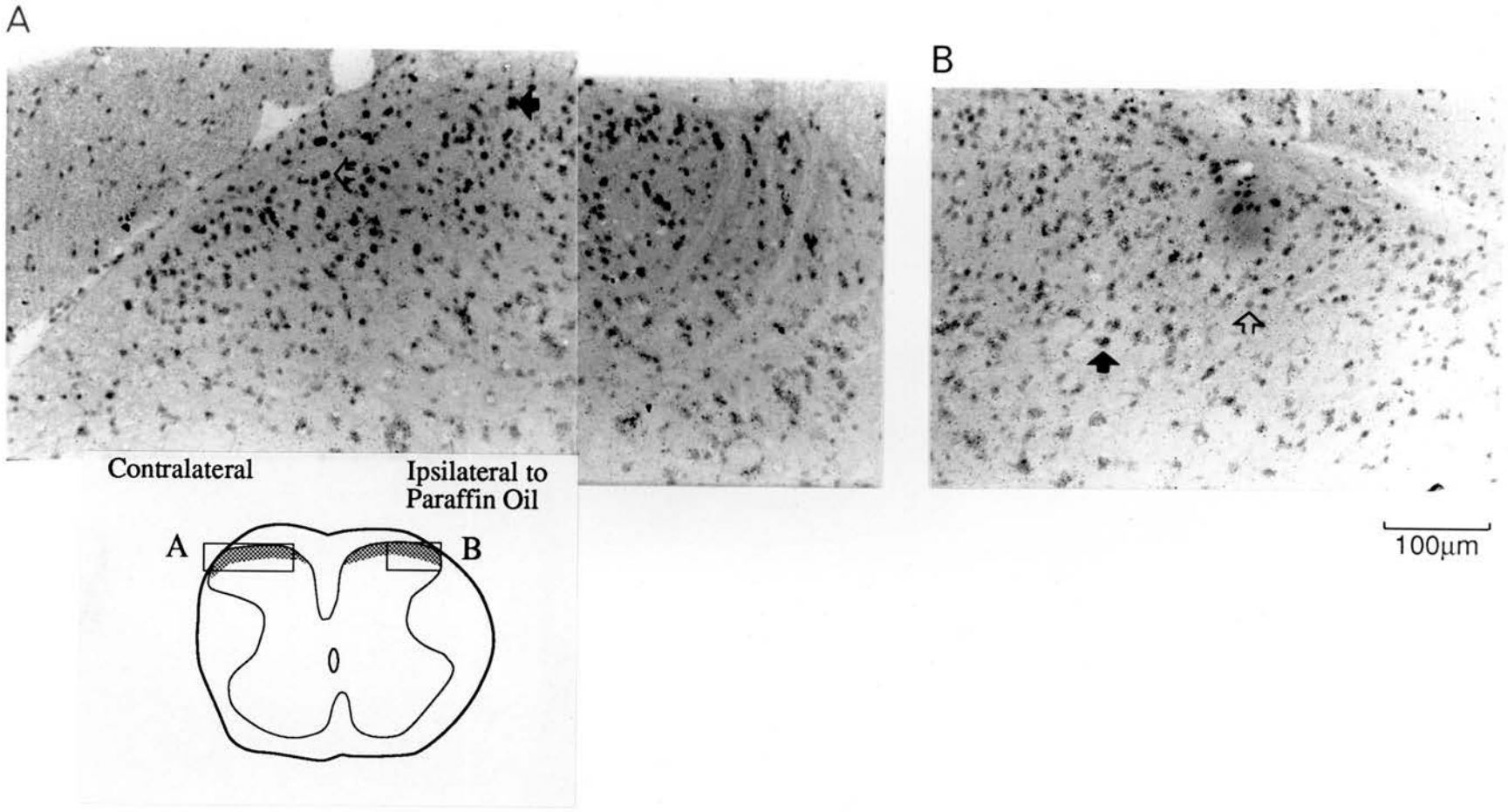

A
B
Contralateral
Ipsilateral to
Paraffin Oil
A
B
100μm

Figure 6.5

Mean density of silver grains (per μm^2) in the superficial and deep dorsal horn following unilateral mustard oil/paraffin oil treatment.

The mean silver grain density per μm^2 was significantly increased in the unilateral mustard oil-treated group compared to the paraffin oil treated group of rats, calculated from a substantial number of sequential sections rostral to caudal, covering segments L 3-4 of the spinal cord. The effects of intravenous drug application were calculated by comparsion of a similar number of sequential sections from SR 48968 and RP 67580 treated rats.

A

Treatment	Number of Sections	Mean Density of Silver Grains (per μm^2) in the Superficial Dorsal Horn Ipsilateral to	
		Treated Limb	Non-Treated Limb
Mustard Oil	135	41.0 ± 2.1	17.1 ± 1.9
Mustard Oil and RP 67580 (1mg/kg)	135	21.1 ± 5.4	23.7 ± 1.6
Mustard Oil and SR 48968 (1mg/kg)	135	21.8 ± 0.8	28.9 ± 1.0
Paraffin Oil	135	6.3 ± 0.3	8.0 ± 0.9

B

Treatment	Number of Sections	Mean Density of Silver Grains (per μm^2) in the Deep Dorsal Horn Ipsilateral to	
		Treated Limb	Non-Treated Limb
Mustard Oil	45	25.8 ± 2.1	20.1 ± 1.3
Mustard Oil and RP 67580 (1mg/kg)	45	25.1 ± 1.4	29.9 ± 2.0
Mustard Oil and SR 48968 (1mg/kg)	45	23.9 ± 1.4	28.9 ± 1.0
Paraffin Oil	45	6.7 ± 1.2	9.0 ± 1.5

Figure 6.6

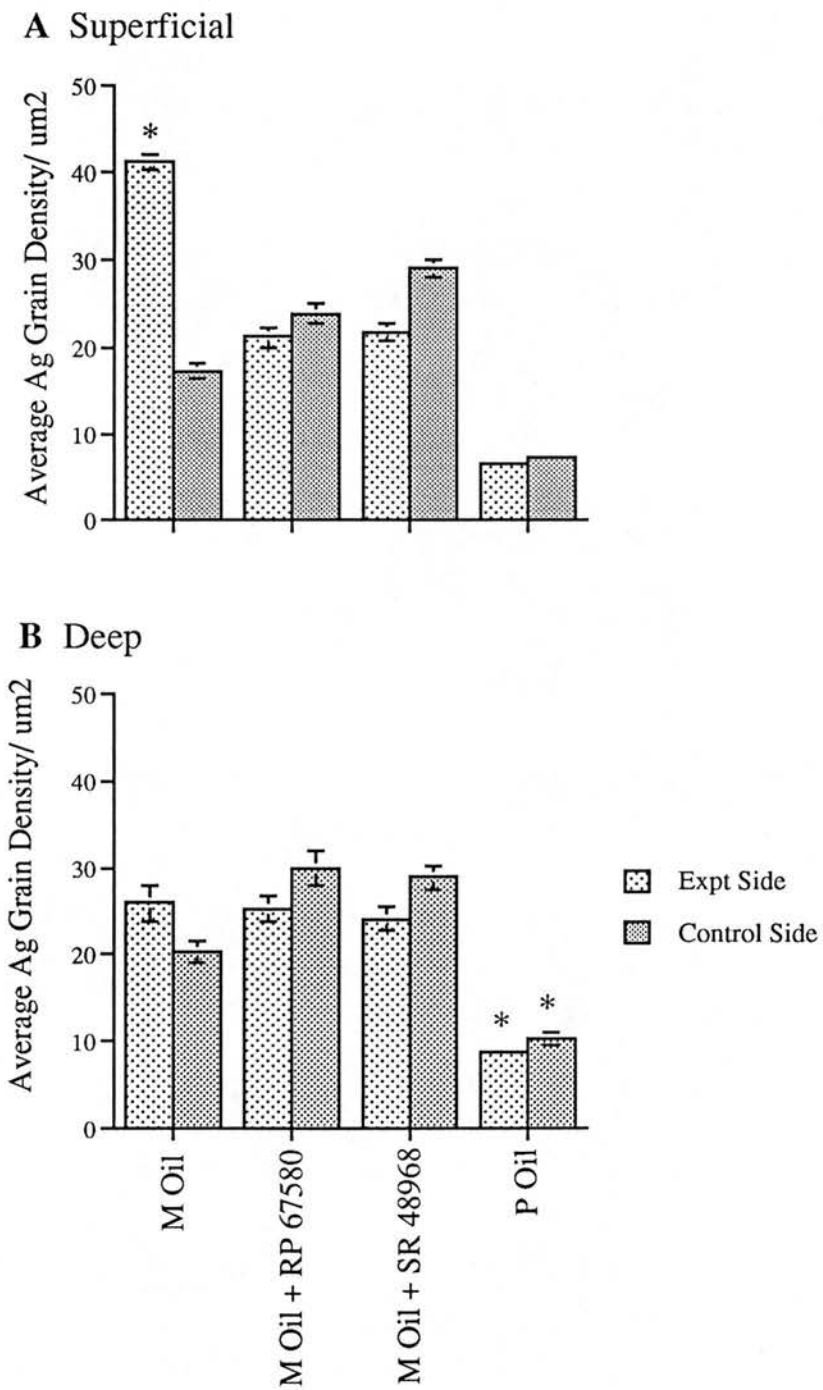
Histogram demonstrating the c-fos mRNA expression in superficial and deep dorsal horn of the spinal cord after unilateral mustard oil application

(A) The silver grain density in the superficial layers of the spinal cord after unilateral application of mustard oil. Unilateral application of mustard oil to the left hindlimb of 3 anaesthetised rats caused a marked facilitation in c-fos mRNA expression on the ipsilateral, experimental side of the superficial dorsal horn as compared to the contralateral, control side. After intravenous injection of the non-peptide NK_1 antagonist, RP 67,580 and the non-peptide NK_2 antagonist SR 48,968, there was a significant decrease in c-fos mRNA expression on the experimental side of the cord compared to the experimental side in mustard oil only treated rats. Interestingly, on the contralateral, control side of the cord (without mustard oil treatment), intravenous administration of both antagonists, resulted in a small but significant increase in c-fos mRNA expression compared to mustard oil only treated rats which had not been given drug. There was no significant difference between the c-fos mRNA expression on ipsilateral and contralateral sides of the RP 67,580 or SR 48,986 treated rats.

(B) In the deeper layers of the spinal cord, unilateral application of mustard oil did not result in such a marked increase in silver grain density as the superficial layers over the time course examined. Treatment with intravenous RP 67,580 or SR 48,968 did not result in any significant change in silver grain density on the ipsilateral side of the cord as compared to the mustard oil only treated rats. However, on the contralateral, control side, after intravenous NK_1 or NK_2 antagonist administration, there is a small, but significant increase in c-fos mRNA expression as compared to mustard oil only.

Statistical significance of the changes was assessed by the Students Matched Paired t-Test for comparisons between ipsilateral and contralateral sides of the same spinal cord and a Students Unmatched Paired t-Test for comparisons between different spinal cords ($*P<0.05$, mean $\pm$ s.e.m.).

C-fos mRNA Expression in Spinal Cord
After Unilateral Mustard Oil.



treated unilaterally with paraffin oil, were examined and the silver grain density appeared as a low and even expression throughout the deeper layers of both sides of the spinal cord.

(c) The Effect of Anaesthetic on Expression of c-fos mRNA

Following unilateral paraffin oil application in a conscious animal, on both sides of the cord there was a dense pattern of silver grain labelling throughout every layer. This suggests that the increase in c-fos mRNA expression compared to anaesthetised control animals, is not substantially due to the treatment of unilateral paraffin oil and is most likely to be due to stress; in agreement with Hayes *et al,* (1976); Rosecrans and Chance, (1976) who have reported that acute exposure to a stressful event results in increased pain thresholds. The pattern of bilateral activation to paraffin oil in conscious rats could potentially mask the true increase in expression caused by mustard oil. It was decided that the conscious preparation would not give a valid estimation of nociception-induced c-fos mRNA expression as it would be difficult to separate this from the stress-induced response.

6.4.3 The Effect of Mustard Oil on c-fos Expression

(a) Peripheral Effects of Mustard Oil

After 15 minutes exposure to topical mustard oil (8%), the painted foot and leg took on a pink and slightly swollen appearance and after a further 30 minutes of exposure, the treated limb appeared to be additionally swollen. There was no marked difference in the appearance of limbs between anaesthetised and conscious animals, however in the latter, behavioural effects indicative of ipsilateral hyperalgesia could also be observed; raising the treated limb to avoid weight bearing.

There was no cross-contamination of mustard oil to the contralateral untreated limb, as the ipsilateral limb was wrapped in cling film and securely bound with adhesive tape, no detectable changes (such as swelling) were observed in the contralateral limb.

(b) Effect of Mustard Oil on c-fos mRNA Expression

Unilateral application of mustard oil to the left hindlimb of 3 anaesthetised rats, caused a marked facilitation of c-fos mRNA expression on the

ipsilateral side of the superficial dorsal horn of the spinal cord, as compared to the contralateral levels (see Figures 6.5, 6.6 and 6.7).

The expression of silver grains was extremely dense in the superficial ipsilateral dorsal horn, therefore individual positive cells could not be accurately resolved. To overcome this, instead of counting positive cells, the silver grain density per μm^2 was measured and the three densest regions of interest in each field (on both control and experimental side) were taken. There was no significant difference between the size of the Regions of Interest (ROIs) on control and experimental sides of the spinal cord. ($P>0.05$).

After unilateral application of mustard oil, c-fos mRNA was found in the same laminar distribution pattern as the control, paraffin oil-treated animals i.e. mainly in the superficial region (Laminae I-II) and deeper in the region of Laminae IV-V. However, in comparison to the paraffin oil treated sections, the mustard oil-treated sections showed a much greater increase in silver grain density, mainly in the lateral edge of the superficial layers of the dorsal horn (see example in Figure 6.7).

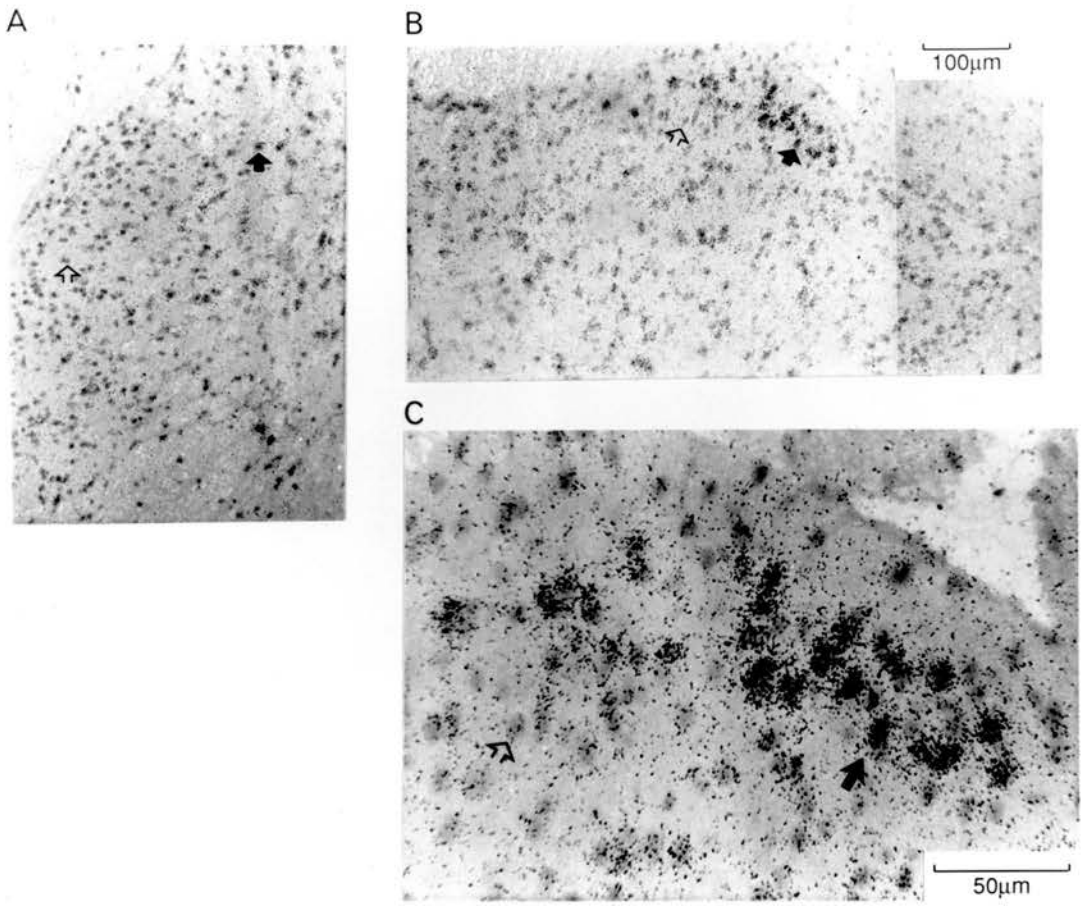
From 3 rats, 135 sections covering lumbar segments L3-4 (within the area of innervation from the hindfoot) were analysed and a mean silver grain density in the superficial layers evaluated (see Figures 6.5 and 6.6). A significant and marked difference in c-fos expression between the control and experimental sides of the spinal cord was found ($P < 0.05$, Matched Pair Students t-test). On the ipsilateral side, the mean silver grain density was 41.0 ± 2.1 per μm^2, more than double that of the mean contralateral control side (17.1 ± 1.9 per μm^2). After unilateral mustard oil application under the present conditions, the density of c-fos mRNA expression in the deeper laminae of the ipsilateral spinal cord was slightly but not significantly different to that on the contralateral side. From a mean of 45 sections it was observed that on the experimental side of the spinal cord there was a mean silver grain density of 25.8 ± 2.1 whilst on the control side it was 20.1 ± 1.3.

A comparison of the results for the anaesthetised mustard oil treated rats was made with those of the anaesthetised control (paraffin oil) treated rats (see Figures 6.4, 6.5 and 6.6). There was a marked and significant (six-fold) increase in the mean silver grain density between mustard oil and paraffin oil-treated rats in both superficial and deep spinal cord. In the superficial spinal cord the mean density on the side ipsilateral to paraffin oil treated side was 6.3 ± 0.3 per μm^2, (mean $\pm$ s.e.m.) and that of the ipsilateral mustard oil treated spinal cord 41.0 ± 2.1 per μm^2, mean $\pm$ s.e.m ($P < 0.05$) ($n=15$), Unpaired Students t-test. When

Figure 6.7

c-fos mRNA expression in the rat lumbar spinal cord following cutaneous mustard oil application.

Low and high power lightfield photographs showing the typical levels of c-fos mRNA expression over the superficial dorsal horn following cutaneous application of mustard oil, as identified by ISHH. Examples of positively-labelled neurons, characterised by a dense aggregation of silver grains around haematoxylin-stained nuclei, are represented by filled arrows. The open arrows demonstrate examples of non-expressing nuclei with low and evenly distributed background density of silver grains. **A** Demonstrates the basal expression, in the side contralateral to the unilateral application of 8% mustard oil. Photographs were taken of overlapping areas and then aligned to show the full mediolateral extent of the superficial region the dorsal horn. **B** Demonstrates the facilitated expression obtained in the opposite dorsal horn, ipsilateral to unilateral application of mustard oil at low-power and **C** demonstrates this facilitated expression at high power.


A
B
100μm
C
50μm
Contralateral
Ipsilateral to
Mustard Oil
A
B

a direct comparison was made between the contralateral sides of both treatments, there was a much smaller but still statistically significant increase in the contralateral side silver grain density (17.1 ± 0.9 per μm^2, mean ± s.e.m.) for the mustard oil-treated rats compared to the paraffin oil-treated rats (8.0 ± 0.9 per μm^2, mean ± s.e.m). $P<0.05$, Unpaired Students t-Test.

In the deep laminae of the spinal cord, a similar pattern of c-fos mRNA expression could be observed (see Figure 6.7). For the ipsilateral spinal cord paraffin oil treated side, the mean silver grain density was 6.7 ± 1.2 per μm^2 (mean ± s.e.m.); whereas the mean silver grain density per μm^2 for the side ipsilateral to mustard oil treatment was significantly increased to 25.8 ± 2.1 per μm^2 (mean ± s.e.m.) $P<0.05$, Unpaired Students t-Test. Similarly when comparing the contralateral, control sides of mustard oil-treated and paraffin oil-treated deep spinal cord, the former is significantly raised (20.1 ± 1.2 per μm^2, mean ± s.e.m.) compared to the paraffin oil treated sections (9.0 ± 1.5 per μm^2, mean ± s.e.m.). $P<0.05$, Unpaired Students t-Test. This implies that there is a smaller contralateral increase due to mustard oil application to the ipsilateral rat hindlimb.

6.4.4 The Effect The Non-Peptide NK_1 Antagonist RP 67580 on Mustard Oil-Induced c-fos Expression.

The effect of intravenously injected RP 67580 (1mg/kg, injected every 15 minutes throughout the experiment lasting a total of 1 hour and 15 minutes) on c-fos mRNA expression was investigated in 3 rats, treated unilaterally with mustard oil as previously described. Comparisons were made firstly between ipsilateral and contralateral sides of corresponding spinal segments. Secondly, a comparison was made between the ipsilateral experimental sides of mustard oil only and mustard oil/ RP 67580-treated spinal segments and a final comparison was made with the control, paraffin oil-treated group of rats.

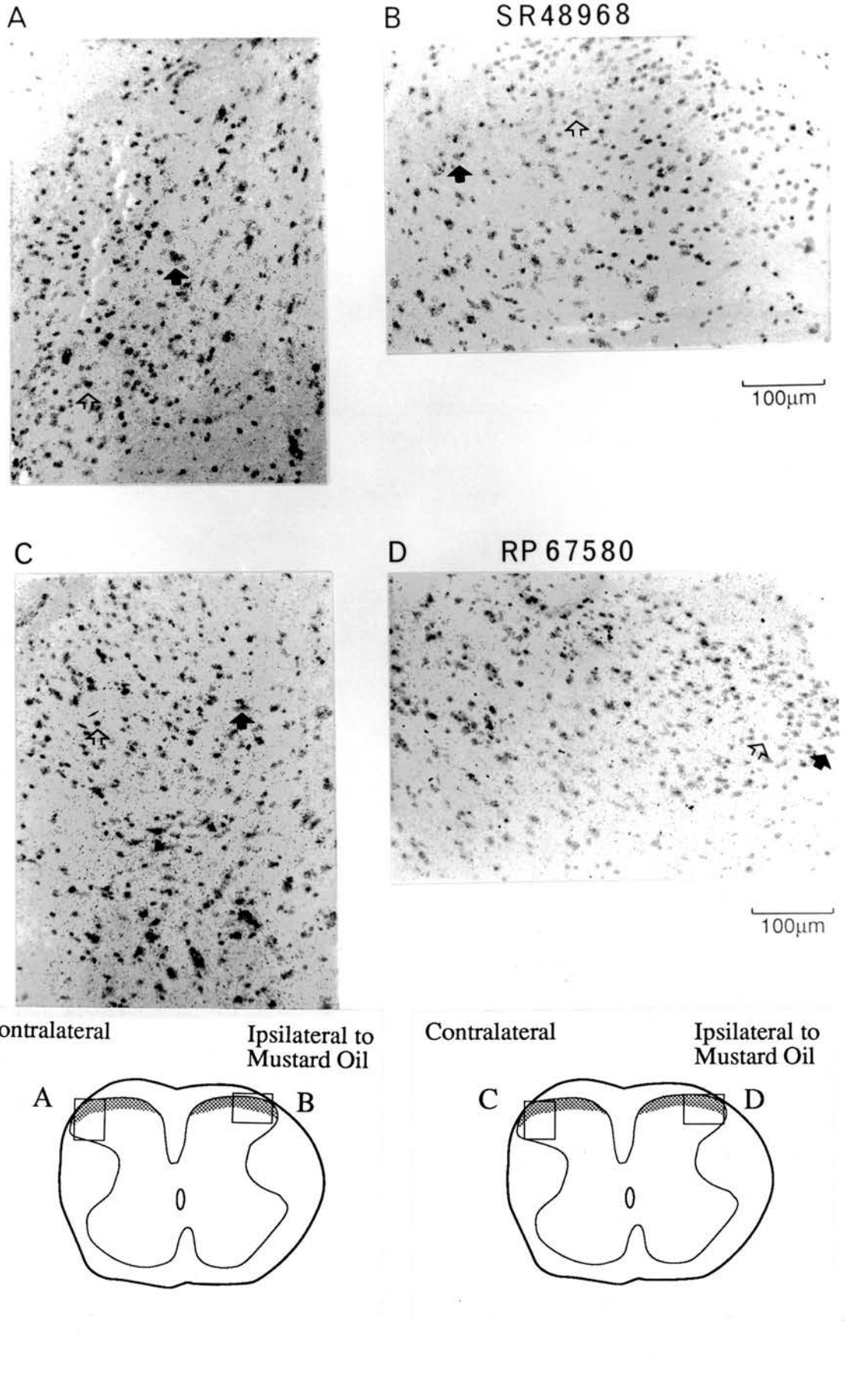
(a) Patterns of c-fos mRNA Expression in Spinal Cord of RP 67580/ Mustard Oil Treated Rats.

After RP 67580 there was no significant difference in c-fos mRNA expression, between the ipsilateral and control, contralateral sides of the same spinal segments in mustard oil-treated rats (see Figure 6.8). The mean expression of c-fos mRNA was estimated from 135 regions of interest and from the table in Figure 6.5 and the histogram in Figure 6.6 it can be seen that in the superficial laminae of the spinal cord, the mean silver grain density on the mustard oil-treated side was 21.1 ± 5.4 per μm^2. There was no significant

Figure 6.8

Photomicrograph examples of the effects of systemically-applied nonpeptide tachykinin antagonists on mustard oil-induced c-fos mRNA expression.

Low power lightfield photographs of examples showing the typical levels of c-fos mRNA expression over the superficial dorsal horn, as identified by ISHH following systemic administration of nonpeptide tachykinin antagonists. Examples of positively-labelled neurons, characterised by a dense aggregation of silver grains around haematoxylin-stained nuclei, are represented by filled arrows. The open arrows demonstrate examples of non-expressing nuclei with low and evenly distributed background density of silver grains. The effects on c-fos mRNA expression are shown for the nonpeptide NK_2 antagonist SR 48968 (**A** and **B**) and the nonpeptide NK_1 antagonist RP 67580 (**C** and **D**). The level of mustard oil-induced c-fos mRNA expression obtained in the presence of these antagonists (**B** and **D**) can be compared with the basal level in their corresponding contralateral sides (**A** and **C** respectively).


A
B
SR48968
100μm
C
D
RP 67580
100μm
Contralateral
Ipsilateral to
Mustard Oil
A
B
Contralateral
Ipsilateral to
Mustard Oil
C
D

increase in density on the control side at 23.7 ± 1.6 per μm^2 (P>0.05, Matched Pair Students t-Test, n=15).

A similar expression pattern was seen upon examination of the deeper laminae of corresponding spinal segments (see Figures 6.5, 6.6. and 6.8). There was no significant difference in c-fos mRNA expression between control and experimental sides of the spinal cord. From a mean of 45 regions of interest within 135 regions of interest, it was observed that for the experimental side of the spinal cord there was a mean silver grain density of 25.1 ± 1.4 per μm^2 (mean ± s.e.m.) whilst on the control side there was a slightly but not significantly greater density at 29.9 ± 2.0 per μm^2 (mean ± s.e.m.). P>0.05, Unpaired Students t-Test.

(b) Comparison Between RP 67580/ Mustard Oil Treated and Mustard Oil Only Treated Groups.

A second comparison was made between the rats treated with mustard oil only and rats treated with mustard oil and the non-peptide NK_1 antagonist RP 67580. In the superficial laminae of the dorsal horn it was seen that in comparison to the mustard oil only group, both the experimental and control sides of the RP 67580 treated group were significantly changed (see Figures 6.7 and 6.8). The mean density of the experimental side of the NK_1 antagonist treated group (21.1 ± 1.0 per μm^2) was markedly and significantly decreased from that of the mustard oil only group (41.0 ± 2.1 per μm^2) (P<0.05, Unpaired Students t-Test). However, when comparing the control sides of the two groups, the results do not follow the same trend; here the mean contralateral density for the RP 67580, mustard oil-treated group (24.0 ± 1.6 per μm^2) was slightly, but significantly increased, as compared to the contralateral side of the mustard oil only treated group (17.1 + 1.9 per μm^2). This suggests that intravenous administration of the non-peptide NK_1 antagonist results in a small general increase in c-fos mRNA expression, whilst specifically inhibiting the increase in c-fos mRNA expression due to mustard oil application.

An entirely different pattern of c-fos mRNA expression was seen upon examination of the deeper laminae of the spinal cord. The mean density of the NK_1 antagonist plus mustard oil treated side was not significantly changed from that of the mustard oil only treated side. However in the deeper laminae, the effect of RP 67580 alone on the density pattern of c-fos mRNA expression seems to mirror the pattern in the superficial laminae with a slight, but significant increase in the silver grain density on the RP 67580 plus mustard oil contralateral control

side (29.0 ± 1.9 per μm^2) compared to the control contralateral mustard oil only-treated animals (23.0 ± 2.7 per μm^2)($P<0.05$, Unpaired Students t-Test).

(c) Comparison Between RP 67580/ Mustard Oil Treated and Paraffin Oil Control Groups.

A final comparison was made between the RP 67580 plus mustard oil treated group of rats and the group treated with paraffin oil only. In the superficial laminae of the dorsal horn it can be seen in Figures 6.4, 6.5, 6.6 and 6.8 that although intravenous treatment with the non-peptide NK_1 antagonist considerably reduced the mustard oil-induced facilitation in c-fos mRNA expression to 21.1 ± 1.0 per μm^2, (mean ± s.e.m.), it did not reduce this expression to the level of the paraffin oil (control) treated rats (6.3 ± 0.2 per μm^2, mean ± s.e.m.). When both the contralateral, control sides of the cord were directly compared, the control side of the mustard oil plus NK_1 antagonist-treated sections had a significantly increased level of c-fos mRNA expression in comparison to the control side of the paraffin oil-treated sections, (23.7 ± 1.1 compared with 7.1 ± 0.3 density per μm^2, mean ± s.e.m.) ($P<0.05$, Unpaired Students t-Test). Therefore, although the intravenous NK_1 antagonist appeared to be inhibiting the mustard oil-induced facilitation of c-fos expression the residual level of expression was still higher than in controls devoid of either noxious stimulation or drug treatment.

A similar situation was observed when deeper laminae are compared under corresponding conditions. From a mean of 45 regions of interest, it was observed that for the mustard oil plus NK_1 antagonist-treated side of the spinal cord there was a mean silver grain density of 25.1 ± 1.4 per μm^2 (mean ± s.e.m.) whilst for the side of cord ipsilateral to just paraffin oil-treatment this was significantly lower at 7.9 ± 0.7 per μm^2 (mean ± s.e.m.). $P<0.05$, Unpaired Students t-Test. When comparing the contralateral, control sides of the two treatments, a similar pattern was seen with the c-fos mRNA level for the paraffin oil treatment having a silver grain density of 10.2 ± 0.8 per μm^2 (mean ± s.e.m.) which was much lower than 29.9 ± 2.0 per μm^2 for the mustard oil plus RP 67580 treatment (mean ± s.e.m.). $P<0.05$, Unpaired Students t-Test.

6.4.5 The Effect of Mustard Oil and The Non-Peptide NK_2 Antagonist SR 48968 on c-fos Expression.

The non-peptide NK_2 antagonist SR 48968 was investigated in a similar manner to RP 67580. Intravenous SR 48968 was administered to unilaterally mustard oil treated rats and its effect on the expression of c-fos mRNA determined

by quantifying the silver grain density on the mustard oil and non-treated sides of the spinal cord. Additionally, the results were compared with the group of rats which had unilateral mustard oil only and also the control group which were unilaterally painted with paraffin oil.

(a) Patterns of c-fos mRNA Expression in Spinal Cord of SR 48968/ Mustard Oil Treated Rats.

After intravenous SR 48968 (at a dose of 1mg/kg every 15 minutes) the results manifest a similar trend as for the NK_1 antagonist RP 67580 (see Figures 6.5, 6.5 and 6.8). In the uppermost laminae of the spinal cord sections analysed, there was no significant difference in mean silver grain density between corresponding experimental (mustard oil) and non-experiment (control) sides. ($P>0.05$, Matched Pair Students t-Test) from the same spinal cord sections i.e. the SR 48968 had prevented laterally-specific differences due to mustard oil. The mean expression of c-fos mRNA was calculated from a total of 135 regions of interest chosen from 3 rats and the results show that after intravenous SR 48968 on the mustard oil-treated side of the cord, the average density of silver grains was 21.7 ± 0.8 per μm^2 (mean $\pm$ s.e.m.) compared to a control side density of 28.9 ± 1.0 per μm^2 (mean $\pm$ s.e.m.)($P<0.05$, Matched Pair Students t Test).

In the deeper laminae of the spinal cord after analysis of 45 regions of interest from 3 rats treated with SR 48968, the control side of the cord showed a slight increase in c-fos mRNA expression to 28.9 ± 1.0 per μm^2 although it was not significantly different from the mustard oil treated side (with a density of 23.9 ± 1.4 per μm^2).

(b) Comparison Between SR 48968, Mustard Oil Treated and Mustard Oil Only Treated Groups.

A second comparison was made between the mustard oil only treated group of rats and the group treated additionally with the highly selective non-peptide NK_2 antagonist SR 48968. From 3 rats, 135 regions of interest in the superficial dorsal horn were analysed for each group and the results show there was a marked difference in the mean silver grain density between the mustard oil treated side for each group (see Figures 6.7 and 6.8). Whereas the mustard oil only group had a mean density per μm^2 of 41.0 ± 1.1, intravenous treatment with SR 48968 caused a marked decrease in expression density to 21.7 ± 0.8 per μm^2. When the contralateral, control sides were compared for each of the two treatments the results were different; here the mean contralateral density for the SR

48968/ mustard oil-treated group (28.9 ± 1.0 per $\mu m^{2)}$ was slightly, but significantly increased as compared to the contralateral side of the mustard oil only treated group (17.1 ± 1.9 per $\mu m^{2)}$. Similar to the intravenous administration of RP 67580, this suggests that intravenous administration of the non-peptide NK_2 antagonist results in a small general increase in c-fos mRNA expression whilst specifically inhibiting the increase in c-fos mRNA expression due to mustard oil application.

Upon examination of the c-fos mRNA pattern in the deeper laminae of the spinal cord a different pattern of c-fos mRNA expression was seen. The mean density of the NK_2 antagonist/mustard oil-treated side was not significantly changed from that of the mustard oil only treated side. However in the deeper laminae, the effect of SR 48968 alone on the density pattern of c-fos mRNA expression seems to mirror the pattern in the superficial laminae with a slight, but significant increase in the silver grain density on the SR 48968/mustard oil contralateral control side (28.9 ± 1.4 per $\mu m^{2)}$ compared to the mustard oil only control (23.0 ± 2.7 per μm^{2})($P<<0.05$, Unpaired Students t-Test).

(c) Comparison Between SR 48968, Mustard Oil Treated and Paraffin Oil Control Groups.

In a similar manner to the NK_1 antagonist RP 67580, the non-peptide NK_2 antagonist, although considerably reducing the mustard oil facilitated expression of c-fos mRNA compared to treatment with mustard oil only, did not result in a drop to the level of the control expression in the presence of ipsilateral paraffin oil treatment (see Figures 6.4 and 6.8).

In the superficial laminae of the cord, in comparison to the ipsilateral side of the paraffin oil only treated animals (with a mean density per μm^2 of 6.3 ± 0.2; mean ± s.e.m.) the SR 48968/ mustard oil-treated sections had a significantly higher density per μm^2 of 21.7 ± 0.8 (mean ± s.e.m.). $P<0.05$, Unpaired Students t-Test. This expression pattern was mirrored in the contralateral sides of both treatments, with a four-fold increase in silver grain density on the mustard oil/ SR 48968 treated segments compared to the paraffin oil only treated segments.

A similar situation was observed when the deeper laminae of the above two treatments were compared. From a mean of 45 regions of interest, it was observed that for the ipsilateral side of the mustard oil/ NK_2 antagonist-treated cord there was a mean silver grain density of 23.9 ± 1.4 per μm^2 (mean ± s.e.m.), whilst on the side ipsilateral to paraffin oil treatment this was significantly lower at 8.5 ± 0.5 per μm^2 (mean ± s.e.m.). $P<0.05$, Unpaired Students t-Test. When

comparing the contralateral, control sides of the two treatments, a similar pattern was seen with the c-fos mRNA level for the paraffin oil treatment having a silver grain density of 10.2 ± 0.8 per μm^2 (mean ± s.e.m.) which was much lower than 28.9 ± 1.0 per μm^2 for the mustard oil/ SR 48968 treatment (mean ± s.e.m.). $P<0.05$, Unpaired Students t-Test.

6.5. DISCUSSION

The results presented here demonstrate that cutaneous application of the chemical algogen mustard oil (known to selectively stimulate C fibres) led to the facilitation of spinal cord c-fos mRNA expression, detected by *in situ* hybridisation histochemistry (ISHH), which was both reproducible and quantifiable, making it suitable for manipulation as a marker.

RNAse treatments clearly demonstrated the specificity of the ISHH technique to selectively identify an RNA sequence, indicating that the ISHH technique employed here was highly likely to be specifically detecting c-fos mRNA expression. A further control study was undertaken by applying paraffin oil to identical areas of the hind-limb and foot as for mustard oil. The results demonstrate a low and even distribution of silver grains spread through the spinal cord (both treated and untreated sides) with no apparent difference between medial and lateral areas of the superficial layers and thus representing a basal c-fos expression. This is in agreement with other studies which have measured c-fos levels in untreated animals (Abbadie and Besson, 1993) or following non-noxious brushing of hairs and gentle manipulation of joints (Hunt *et al*, 1987; Williams *et al*, 1989). In the latter studies, following continuous non-noxious brushing and gentle manipulation of joints for a period of 15 minutes, labelled nuclei were seen mainly in layers II to V and rarely in layer I, consistent with actions mediated from the terminals of low-threshold Aδ and C primary afferents (Light and Perl, 1977, 1979). However, a few labelled neurons in lamina I were observed here, which may be explained by either the differences and time scales of the techniques employed or the low numbers employed in each set of experiments. Further influencing factors may be the anaesthetics used and level of surgery employed in each study (see Hunt *et al*, 1987) or stress-induced c-fos expression suggested by Sharp *et al*, (1991).

Following cutaneous application of mustard oil to the hind-foot and limb of the rat, these ISHH studies demonstrate a marked increase in c-fos mRNA expression in the superficial laminae of the ipsilateral dorsal horn compared to the

contralateral side of the dorsal horn. The expression of silver grains was particularly dense on the lateral edge of the ipsilateral superficial dorsal horn of lumbar segments 3-5, as is appropriate for this cutaneous input. Most labelled nuclei were found in layers I (marginal) and II (substantia gelatinosa) where the majority of unmyelinated afferents terminate (Molander and Grant, 1986), but c-fos labelled nuclei were also found in layers IV-V and occasionally in layers VI and VII and around the central canal (layer X).

The general pattern of this facilitation was in agreement with other studies on the mustard oil-induced facilitation of c-fos mRNA expression in rat lumbar spinal cord. Although no other groups have used similar ISHH techniques following mustard oil-induced noxious stimulation, the distribution pattern of cells expressing c-fos mRNA, closely followed that found previously by other groups using immunohistochemistry techniques (Hunt *et al*, 1987; Williams *et al*, 1989; Pretel and Piekut, 1991). However, in comparison to the present results (and those of Pretel and Piekut, 1991) where the principal increase in c-fos mRNA expression was on the lateral edge of the ipsilateral dorsal horn, others reported P^{55}c-fos-like immunoreactivity was localised to nuclei in a restricted region of the ipsilateral medial dorsal horn (Hunt *et al*, 1987). One possible explanation is the variability of the precise site of mustard oil application between studies, whereas we applied the algogen to the entire foot and hind-limb, Hunt *et al* applied it to an unspecified region of the hind-limb only.

These experiments established the suitability of using the contralateral dorsal horn as an internal standard to compare possible effects of tachykinin antagonists in later experiments. In these experiments the facilitated c-fos mRNA expression remained generally ipsilateral to the cutaneous mustard oil application, although compared to paraffin oil there was a small but significant increase in c-fos mRNA expression on the contralateral side of the spinal cord following peripheral application of the chemical algogen, entirely in accord with the findings of other groups (Hunt *et al*, 1987; Williams *et al*, 1989; Pretel and Piekut, 1991). This might be due to a small group of primary afferents which has been shown to terminate in the contralateral superficial dorsal horn (Light and Perl, 1979) or alternatively stress-induced (Sharp *et al*, 1991) or anaesthetic and surgery-induced c-fos mRNA expression (Hunt *et al*, 1987).

Mustard oil application led to visibly obvious redness and swelling as reported to be indicative of development of inflammation, central sensitisation and hyperalgesia (Treede *et al*, 1992). Other inflammatory or sustained C fibre stimulation models have been used to investigate the facilitation of central c-fos

mRNA expression. Following formalin- or carrageenan- induction of short-term inflammation and hyperalgesia, a very rapid increase in the levels of c-fos mRNA or Fos protein has been demonstrated in a somatotopic representation of the particular nociresponsive neurons stimulated within the dorsal horn (Draisci and Iadarola, 1989; Williams *et al*, 1989; Presley *et al*, 1990; Bullit, 1991; Noguchi *et al*, 1991,1992; Leah *et al*, 1992). Similarly, the expression of c-fos and Fos has also been demonstrated in rat spinal dorsal horn consistent with the known nociceptive primary afferent input from the knee following induction of the longer-term hyperalgesic state of adjuvant-induced arthritis (Menetrey *et al*, 1989; Hylden *et al*, 1992; Abbadie and Besson, 1993; Tolle *et al*, 1994) and in addition, the development of a neuroma following nerve injury (Chi *et al*, 1993).

The tachykinins SP and NKA are two of several transmitters which might provide input to modulate and regulate the expression of c-fos. SP is known to be present in small diameter primary afferents (Hokfelt *et al*, 1975b) and after cutaneous mustard oil application, which stimulates small diameter afferents, the majority of c-fos expressing neurons, in laminae I and II of the lumbar spinal cord also received input from SP immunoreactive neurons (Pretel and Piekut, 1991). As SP and NKA are derived from the same gene (Nawa *et al*, 1984), are thought to be co-localised and are distributed in a similar manner throughout the spinal cord (Ogawa *et al*, 1985), it is also feasible that NKA may play a role in regulation of expression of c-fos in the spinal cord. Indeed, following intrathecal perfusion of SP or NKA into the sub-arachnoid space, P^{55}c-fos was generated in the superficial laminae of the dorsal horn (Williams *et al*, 1989). Therefore the exclusive mustard oil-facilitated expression of c-fos, established as a marker, was used to investigate the effects of intravenous administration of non-peptide NK_1 and NK_2 tachykinin antagonists on the spinal changes accompanying cutaneous application of mustard oil.

The results presented here indicate that both the NK_1 and NK_2 receptors accessed here appear to be involved in mediating mustard oil-induced c-fos mRNA expression within the superficial dorsal horn of the spinal cord. Systemic application of antagonists to either of these receptor types lead to a significant inhibition, to very similar levels, in the mustard oil-induced expression of specifically hybridising material.

RP 67580 is reported to be a highly-selective and potent non-peptide antagonist for the rat NK_1 receptor (Garret *et al*, 1991; Petitet *et al*, 1993), indicating that the results obtained with this drug are likely to represent selective NK_1 mediated actions, although unknown side-effects cannot be ruled out. In the

present studies, RP 67580 administration resulted in a significant decrease in the mustard oil-evoked facilitation of c-fos mRNA expression on the ipsilateral side of the spinal cord in comparison to the mustard oil only-treated group of rats. However, when a comparison is made between the control sides of the two groups, the results do not follow the same trend, the mean contralateral density for the RP 67,580 mustard oil-treated group was slightly, but significantly increased compared to the contralateral side of the mustard oil-treated group. This suggests that intravenous administration of the non-peptide NK_1 antagonist results in a small general increase in c-fos mRNA expression, whilst specifically inhibiting the increase in c-fos mRNA expression due to mustard oil application. This result may possibly indicate opposing roles for different NK_1 receptor sites in this mustard oil-induced cellular response, although it is possible that this may be a vehicle-induced effect and ideally we should have been carried out a set of vehicle or respective enantiomer controls using RP 68651, which reportedly shows no significant affinity for the NK_1 binding site (Garrett *et al*, 1991).

Although intravenous administration of RP 67580 considerably reduced the ipsilateral mustard oil-induced facilitation in c-fos mRNA expression, it did not reduce this expression to the level of the ipsilateral paraffin oil- treated animals. Moreover, the control side of the mustard oil plus NK_1 antagonist-treated sections had a significantly increased level of c-fos mRNA expression in comparison to the control side of the paraffin oil-treated sections. Therefore, although the intravenous NK_1 antagonist appeared to be inhibiting the mustard oil-induced facilitation of c-fos expression, the residual level of expression was still higher than in controls devoid of either noxious stimulation or drug treatment. It may be that other mechanisms or neuropeptides are involved or alternatively that the full extent of the drug effect is not apparent as RP 67580 is known to have a short duration of action (Laird *et al*, 1993). Although repeated doses of drug were administered every 15 minutes, perhaps the full effect of the selective NK_1 antagonist was not being seen as it has been reported that RP 67580 has a low penetration in the rat CNS (Fardin *et al*, 1993). However, there certainly was an effect in this study following repeated doses of RP 67580 and also following a single application on the nociceptive rat flexor reflex (Laird *et al*, 1993) on SP-induced excitation of locus coerulus neurons (Allam *et al*, 1992) and on SP-induced extravasation in the dura mater (Moussaoui *et al*, 1993).

These results are entirely consistent with the results obtained in both electrophysiological and behavioural studies (see Chapters 2,3,4 and 7) in addition to a number of other studies which have also revealed that NK_1 receptors may

have an important role in mediating sustained nociceptive responses but appear to be less involved in acute nociception. During the formalin or CFA-induced inflammation there are increases in the biosynthesis of SP-IR and PPT-1 mRNA and expression of NK_1 gene expression and NK_1 binding sites in the spinal cord dorsal horn (Minami *et al*, 1989; Donaldson *et al*, 1992; Noguchi and Ruda, 1992; Schafer *et al*, 1993; Stucky *et al*, 1993; McCarson and Kruase, 1994). Increased release of SP-IR into the spinal dorsal horn occurs in models of inflammation and sustained C fibre activity (Go and Yaksh, 1987; Oku *et al*, 1987; Kuraishi *et al*, 1989; Schaible *et al*, 1990; McCarson and Goldstein, 1991; Garry and Hargreaves, 1992) and the presence of CGRP can act to maintain the levels of SP and promote its spread through the dorsal horn (Schaible *et al*, 1990). Just as these changes suggest, there is compelling evidence that NK_1 receptors play an increasing role in nociception following inflammation. NK_1 antagonists are highly effective analgesic agents in a variety of behavioural models of sustained and inflammatory pain (Garrett *et al*, 1991; Yamamoto and Yaksh, 1991; Birch *et al*, 1992; Nagahisa *et al*, 1992; Chapman and Dickenson, 1993; Yashpal *et al*, 1993) and although they are not effective in all cases (Nagahisa *et al*, 1992; Rupniak *et al*, 1993), individual drug characteristics may contribute to those particular finding. The secondary (contralateral) hyperalgesia which develops following heat injury to one hindpaw of rats, is similarly reversed by an NK_1 antagonist (Murray *et al*, 1991). NK_1 agonists and conditioning stimulation of the sural nerve both elicit facilitation of the nociceptive flexor reflex that is sensitive to selective NK_1 antagonists (Xu *et al*, 1991) pointing to a contribution of NK_1 receptors to the central sensitisation following sustained C-afferent input. Equally, SP was able to enhance and a NK_1 receptor antagonist inhibit the responses of nociceptive dorsal horn neurons to repetitive C fibre strength stimulation (Kellstein *et al*, 1990) in addition to the underlying long-duration postsynaptic dorsal root potentials (Urban and Randic, 1984). In the ultraviolet burn model, using an *in vitro* spinal cord-skin preparation (Thompson *et al*, 1994), the NK_1 receptor antagonist CP-96,345 was substantially effective only in the inflamed condition.

SR 48968 has been demonstrated to be a highly potent and selective antagonist of NK_2 receptors (Advenier *et al*, 1992; Emonds-Alt *et al*, 1992,1993). Systemic administration of this selective non-peptide NK_2 antagonist resulted in a very similar outcome to that achieved following administration RP 67580. Following intravenous administration of SR 48968 there was a significant decrease in the ipsilateral mustard oil-induced facilitation of c-fos mRNA expression. However in contrast, SR 48968 increased the number of c-fos

mRNA-expressing superficial dorsal horn neurons contralateral to unilateral mustard oil application as compared to the mustard oil only group of controls. As previously suggested, possible explanations for this spurious result are either a vehicle-induced effect or opposing roles for different NK_2 receptor sites in this mustard oil-induced cellular response. An alternative suggestion may be that there are non-specific actions of SR 48968, not related to its action at NK_2 receptors. Indeed, in guinea pig tissue SR 48968 is reported to have activity at NK_3 receptors although in the rat cortical tissue it appears to remain fairly selective for NK_2 receptors (Petitet *et al*, 1993). However, μ agonist actions at higher concentrations of SR 48968 have been indicated (Martin *et al*, 1993), although it is not clear how μ agonist actions could increase the c-fos mRNA expression within spinal cord neurons. Despite this overall increase in c-fos mRNA expression on both sides of the cord there was a definite and selective inhibition of the mustard oil-induced facilitation of silver grain density suggestive of an NK_2 receptor involvement in this sustained activation of C-fibres following mustard oil activity.

These results are in close agreement with the electrophysiological, biochemical and behavioural results discussed in this Thesis. There are also a variety of other reports indicating that NK_2 receptors are extremely important in mediating sustained spinal nociceptive processing. Adjuvant or formalin-induced inflammation and hyperalgesia lead to an increased biosynthesis of PPT-1 mRNA (Minami *et al*, 1989; Donaldson *et al*, 1992) and increased release of NKA was seen following kaolin and carrageenan injection into the knee joint (Hope *et al*, 1990). Electrophysiological studies demonstrate a role for NK_2 receptors in mediating the flexor reflex facilitation evoked by damaging cutaneous thermal stimuli (Xu and Wiesenfeld-Hallin, 1992) or following gastrocnemius nerve conditioning at C fibre strength (Xu *et al*, 1991). Correspondingly, in the *in vitro* UV burn model, the C-fibre evoked ventral root potential was significantly reduced by a selective NK_2 antagonist both pre- and post-inflammation again providing evidence that NK_2 receptors are important in prolonged nociceptive states.

Our observations indicate that Fos protein is probably induced at least in part through actions of the neuropeptides SP and NKA. These tachykinins are known to be present in small diameter afferents (Hokfelt *et al*, 1975b) which are the major inputs to these regions of greatest c-fos mRNA expression in the spinal cord whose laminar distribution is related to the nature of the sensory stimulus. The significance of the induction in c-fos is unclear, but it is suggested that it is a rapid response to injury of peripheral tissue and may contribute to long-term

adaptations of these neurons conceivably through an increase in dynorphin synthesis. There is strong evidence suggesting that the preprodynorphin gene is a target for Fos as co-induction of c-fos and preprodynorphin genes is reported to occur in the same neuronal elements of the spinal cord after noxious thermal stimulation (Naranjo *et al*, 1991; Draisci and Iadarola, 1989) and inflammation (Draisci and Iadarola, 1989; Hylden *et al*, 1992). The increase in Fos protein after peripheral inflammation is followed by a large increase in preprodynorphin mRNA and a subsequent increase in dynorphin peptide (Iadarola *et al*, 1988). Over 80% of preprodynorphin expressing neurons in dorsal horn co-localise c-fos mRNA (Dubner and Ruda, 1992) and in addition, an AP-1 site has been identified in the preprodynorphin promotor that binds fos/jun proteins (Naranjo *et al*, 1991) providing further support that Fos-mediated signalling is coupled to dynorphin gene transcription. As dynorphin-expressing neurons are thought to be involved in mechanisms that modulate pain sensations (Iadarola *et al*, 1988) and it seems likely that the early induction of c-fos mRNA expression in these cells may fulfil some role in mediating those responses, such changes may underpin persistent changes in the sensitivity of nociceptive processing pathways during inflammation and be integral to the development of lasting hyperalgesia and allodynia. Moreover, following systemic administration of selective NK_1 or NK_2 antagonists, ISHH studies have demonstrated a contribution of both NK_1 and NK_2 receptors to the carrageenan-induced expression of preprodynorphin mRNA in superficial dorsal horn neurons (R.M.C. Parker, unpublished observations).

The precise mechanism through which neurons receive information signalling the necessity for c-fos production and an increase in dynorphin synthesis is unclear. However, as both SP and NKA are known to be contained in small diameter primary afferents (Hokfelt *et al*, 1975b) and are released upon C fibre stimulation (Duggan *et al*, 1987,1988,1990; Hope *et al*, 1990), following sustained C fibre activity, these axons could convey the information from the periphery to spinal cord neurons, depolarise the target neurons and thus induce the expression of c-fos. Through binding to the AP-1 consensus recognition sequence, Fos might induce changes in DNA transcription, which could result in changes of mRNA output and thus changes in synthesis of a peptide such as dynorphin.

Nevertheless, the tachykinins SP and NKA are not the only neurotransmitters which may be involved in the transcriptional activation of c-fos. Although the evidence is somewhat mixed, it is possible that c-fos is induced in the spinal dorsal horn following Ca^{2+} entry through NMDA receptor-operated

Ca^{2+} channels. It has been demonstrated that noxious stimulation-induced expression of Fos in the spinal dorsal horn is substantially reduced by pre-treatment with the NMDA receptor antagonist MK-801 (Kehl *et al*, 1991), however others have found that NMDA antagonists do not affect the distribution of Fos-labelled neurons in spinal cord (Tolle *et al*, 1991). Alternatively it is possible that the c-fos-containing neurons might receive input from several transmitters or from a combination of them depending on the type of stimulation e.g. noxious or innocuous, indeed Pretel and Piekut (1991) have demonstrated co-localisation of c-fos labelled nuclei with enkephalin, SP or 5-HT immunoreactive fibres.

CHAPTER 7:
Behavioural Experiments

7.1 AIMS

The aim of these experiments was to assess the effects of tachykinin antagonists on behavioural paradigms of nociception. To do this, the behavioural responses to rat paw-flick and tail-flick tests were evaluated and these results were used as 'baseline responses'. The effects of intrathecal administration of NK_1 and NK_2 tachykinin antagonists were then assessed in both non-carrageenan treated rats and those treated with an intraplantar injection of carrageenan (a peripheral inflammatory stimulus).

7.2 MATERIALS

Male Lister hooded rats were obtained from Glaxo Laboratories, Ware, Herts, UK. All standard laboratory equipment and chemicals which were used in these studies were also from Glaxo Laboratories. L-659,874 was purchased from Cambridge Research Biochemicals; GR 82334 was a gift from Glaxo Group Research; SR 48968 was a gift from Sanofi Recherché, France and RP 67580 was a gift from Dr C Garret, Rhone Poulenc, France.

7.3 METHODS

7.3.1 Animals

Rats weighing between 40-80g were used for the intrathecal route of injection. All animals were housed in suspended steel cages in a colony room maintained on a 12 hour light : 12 hour dark cycle. Experiments were conducted at the same time each day. Food and water were available ad libitum throughout the experiments.

7.3.2 Drug Administration

Drugs were made up in either sterile saline or DMSO on the day of use. The pH of each drug solution was adjusted to as near 7.0 as possible and concentrations were made up such that the micro-injection volume of 10µl

contained the appropriate drug amount. When 2 drugs were co-administered, the combination was in a single solution. The following drugs were administered by this route:- L-659,874 (2,6 and 60nmol, pH 6.5-7.0, prepared from concentrated stocks dissolved in DMSO (0.5% final concentration)); GR 82334 (2,6 and 60nmol, pH 6.5-7.0, prepared from concentrated stocks dissolved in saline). The drugs were made up as stock solutions, stored at -20°C over dessicant. Solutions once thawed were not re-frozen or used again. Vehicle controls used were either sterile saline or 0.5% DMSO.

An intrathecal injection was used to administer both the peptide compounds and vehicle controls using the standard protocol of Hylden and Wilcox, (1980) (see Figure 7.1). All intrathecal injections were performed with disposable 30 guage 1/2-inch needles mated to a 10µl luer tip syringe. The injection was initially performed using the highly selective NK_1 agonist SPOMe, known to cause dose-dependent biting and scratching behaviour following its intrathecal administration (Gamse and Saria, 1986). When the experimenter was proficient at delivering this NK_1 agonist accurately (assessed by the ensuing biting and scratching behaviour), the injection of tachykinin antagonists could be initiated. The rat was held firmly by the pelvic girdle in one hand, while the syringe was held in the other hand at an angle of about 20° above the vertebral column. The needle was inserted into the tissue to one side of the L5 or L6 spinous process so that it slipped into the groove between the spinous and the transverse processes. The needle was then moved carefully foreword to the intervertebral space as the angle of the syringe was decreased to about 10°. The tip of the needle was inserted so that approximately 0.5 cm was within the vertebral column, the solution was injected and the needle rotated on withdrawal. The site of injection in these experiments was chosen to be between L5 and L6. This site represented a compromise to maximise intervertebral accessibility and to minimise the possibility of spinal damage. Cursory examination of injected rats showed no evidence of overt motor impairment.

7.3.3 Nociceptive Testing in Non-Carrageenan Treated Animals

Care was taken to ensure that the rats were not overly stressed; this was achieved by always leaving the rats for at least 30 minutes post transfer to the laboratory and handling the animals for a minimal period.

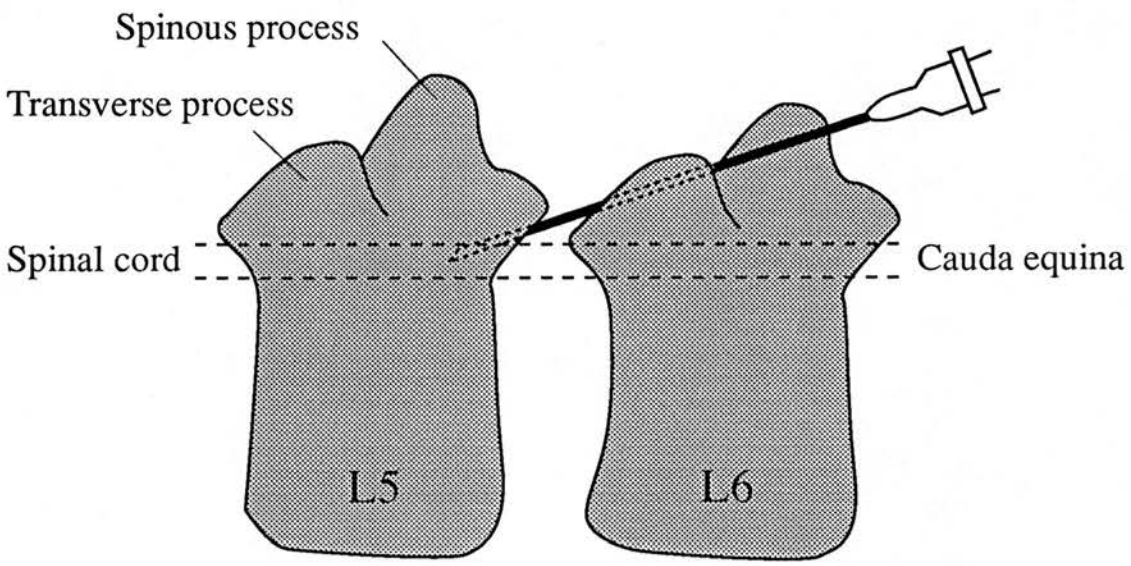
The rats were placed in groups of 6, for each dose of the drug to be tested and their tails pen-marked from 0 to 5 respectively. Each group of six were kept separately in marked cages between tests. Before injection, each rat was

Figure 7.1

Schematic Diagram of the Intrathecal Injection.

Diagram showing insertion of the needle into the invertebral; space between L5 and L6.

(From Hylden and Wilcox, 1980)


Spinous process
Transverse process
Spinal cord
Cauda equina
L5
L6

consecutively tested, first for tail-flick and then paw-flick, this was repeated three times and the values were averaged for baseline control values. The animals were then injected in the same order and the nociceptive tests carried out at 1, 3 and 5 minutes post injection.

Two methods of nociceptive testing were used:-

(a) Tail-flick Test

A Ugo Basile tail-flick unit was used for these studies. This consists of an infrared (IR) source (50W bulb) whose radiant heat energy of adjustable intensity is focused by an embodied parabolic mirror on the rat tail.

The tail-flick test was carried out following a standard protocol of D'Amour and Smith, (1941), whereby the rat was held loosely on the upper panel (see Figure 7.2) in such a way that its tail, placed over a flush mounted window received the IR energy. The tail was always placed in exactly the same marked position prior to testing with the heat stimulus.

When the rat flicked its tail away from the beam, a sensor mounted coaxially to the infra-red beam detected the lack of energy reflected back by the tail. Via an electronic circuit, this stopped the second counter and switched off the bulb. Thus the time from the onset of this thermal stimulus to the reflex flick of the tail away from the light source, was recorded automatically and termed the tail-flick latency (TFL). An automatic cut-off circuit was provided, which operated either if the tail was misplaced or moved sideways and thus there was no reflected energy or, if the animal did not have a tail-flick reaction within the maximum selected reaction time (23 seconds from initiation of the thermal stimulus).

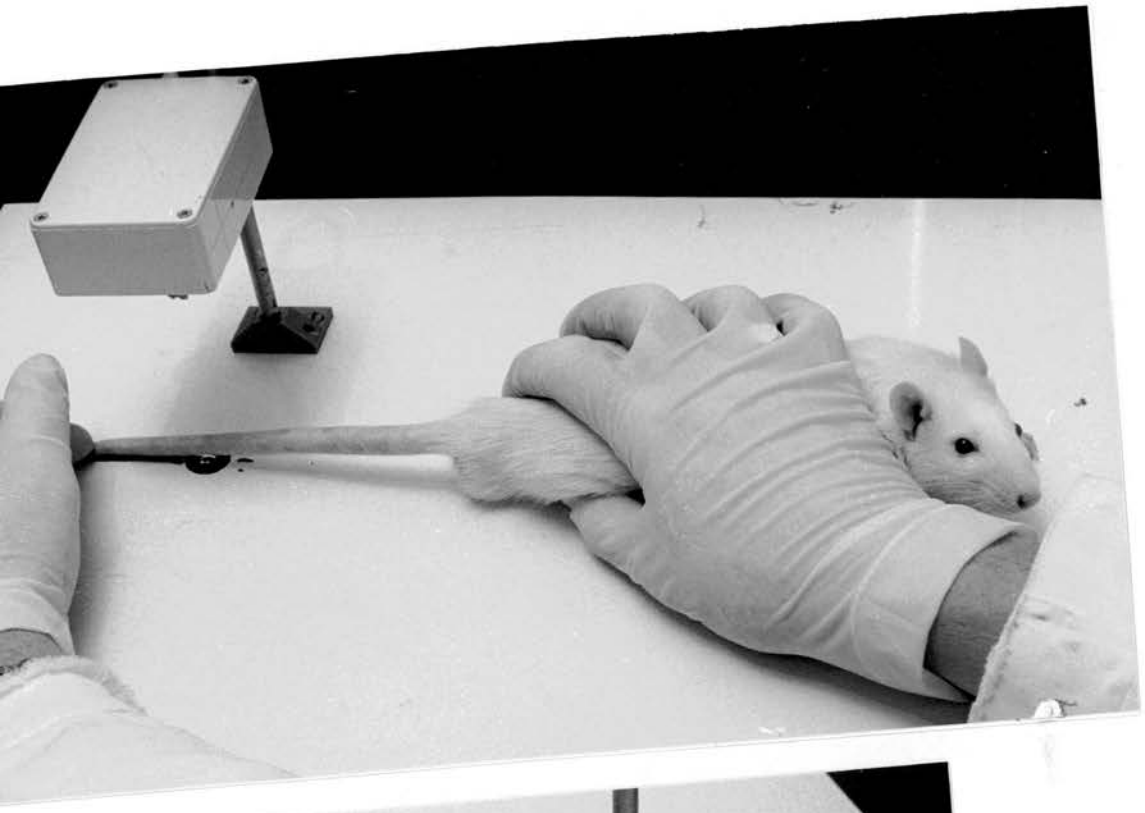
To test the tail-flick, the rat was restrained in a crouching position under the cup of the examiner's hand. The tail was held over the infrared source by loosely holding the end, this allowed any movement of the tail to be sensed by the examiner. The rat either raised its tail away from the light or moved it to one side of the beam when it felt discomfort. However, care had to be taken to ensure that the examiner could distinguish between restless movements of the animal and movement of the tail due to discomfort.

A 2 minute gap was left between tests to ensure that tissue damage to the tail and possible ensuing sensitisation of the tail-flick response did not occur, during this period the animal was placed back in its cage with access to food and water.

Figure 7.2

The rat tail-flick response.

The upper photograph demonstrates positioning of the rat's tail over the IR beam. The rat was held loosely and the tail positioned in such a was that its tail, placed over a flush mounted window received the IR energy. The tail then rested freely over the beam (the lower photograph) and was flicked away when the animal felt discomfort.



(b) Dorsal Paw-flick Test

The dorsal paw-flick test was performed by modification of the Woolfe and MacDonald, (1944) method on the same Ugo Basile Apparatus as the tail-flick tests. The examiner held the rat in an elevated position with the left hindlimb free for testing. The foot was placed over the light source with the paw bent under in such a manner that the infrared beam was focused on the hairy (dorsal) side of the foot. Care had to be taken that the foot was only slightly bent so that the paw could be easily flicked away (see Figure 7.3). A foot operated control was pressed and when the animal flicked its foot away from the noxious heat source in a brisk withdrawal type movement, the reaction time was recorded. Thus the time from the onset of this thermal stimulus to the reflex flick of the paw away from the light source, was recorded automatically and termed the dorsal paw-flick latency (DPFL). Again the automatic cut-off point was set to 23.0 seconds from initiation of the thermal stimulus to avoid damage to the foot if a paw-flick did not occur.

7.3.4 Nociceptive Testing in Carrageenan Treated Animals

The tachykinin antagonists were also tested in rats unilaterally treated with 2% carrageenan. In the same protocol as the non-carrageenan treated groups, the rats were placed in groups of 6 for each dose of the drug to be tested. Before carrageenan injection, each rat was consecutively tested, first for glabrous and then dorsal paw-flick, this was repeated three times and the values were averaged for baseline control values. The animals were then unilaterally injected with carrageenan:

Carrageenan Injection

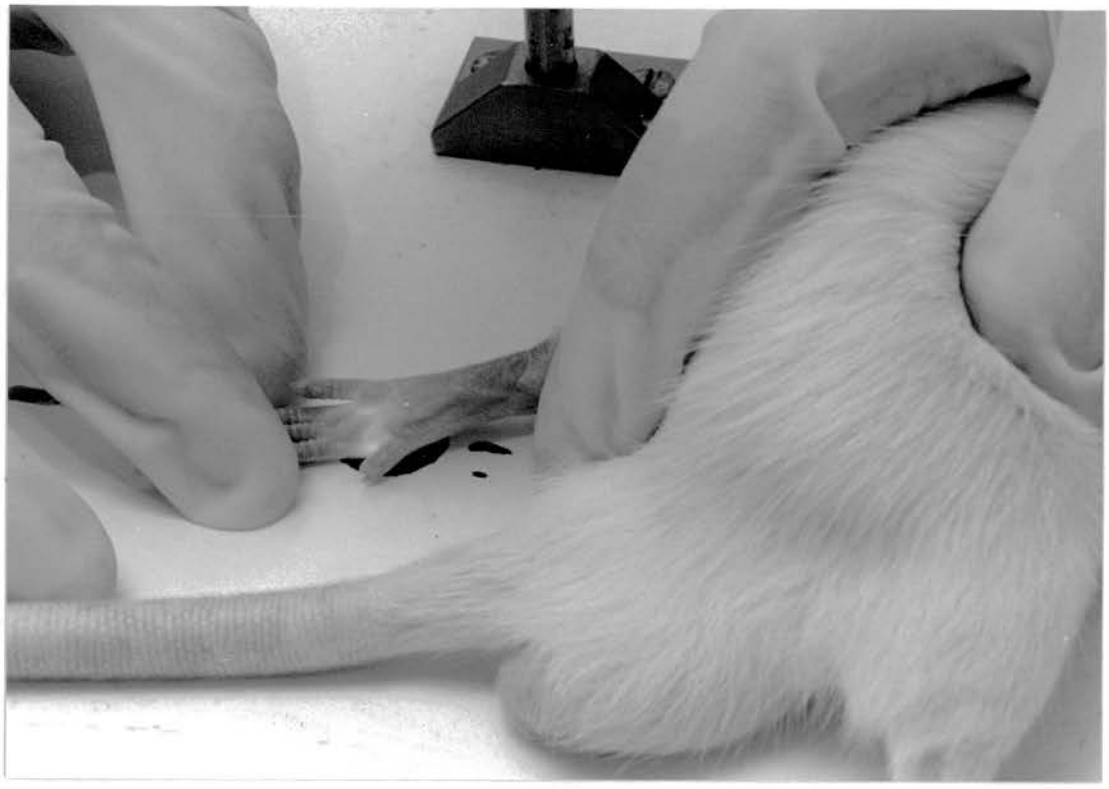
A 2% solution of lambda carrageenan (Sigma) was made up in sterile saline and left at 4°C overnight. Under halothane anaesthesia, the left hindpaw of each animal was injected with carrageenan. The injection was administered under the dorsal (hairy) surface of the foot by carefully sliding the needle under the skin surface and injecting 100µl of the 2% solution slowly to avoid any leakage from the puncture site. The rats were then placed in recovery cages for a minimum period of 3 hours to allow the inflammation to develop.

Once the inflammation had developed (3-4 hours post-carrageenan), the paw-flick tests were completed in triplicate and the shift in baseline controls determined. The animals were then intrathecally injected and the following nociceptive tests carried out at 1, 3 and 5 minutes post injection.

Figure 7.3

The dorsal paw-flick response.

The dorsal paw-flick response was performed on the same apparatus as the tail-flick response. The rat was held in an elevated position and its paw bent under in such a manner that the infrared beam focused on the hairy side of the foot. The upper photograph demonstrates the positioning of the foot in this way. The paw was then unrestrained (the lower photograph) so that the animal was free to flick it away from the IR beam.



(a) Dorsal Paw-flick Tests

The dorsal paw-flick tests were carried out in an identical procedure to that described for the non-carrageenan nociceptive tests (see Figure 7.3).

(b) Glabrous Paw-flick Tests

The rat was held firmly, but gently in one hand with its forelimbs raised, permitting a free range of movement in the rear quarters of the rat. The foot was placed over the light source in such a manner that the infrared beam was focused on the most padded part of the foot and thus presented minimal damage (see Figure 7.4). A foot operated control was pressed and when the animal flicked its foot away from the noxious heat source in a brisk withdrawal type movement, the reaction time was recorded. Thus the time from the onset of this thermal stimulus to the reflex flick of the paw away from the light source, was recorded automatically and termed the glabrous paw-flick latency (GPFL). This reflex had to be discerned from other movements of the rat, such as wriggling or exploratory type movements. The automatic cut-off point was set to 23.0 seconds to avoid damage to the foot if a paw-flick did not occur.

7.3.5 Analysis

Paw-flick and tail-flick latencies were calculated in seconds for each rat under study using the Ugo Basile Apparatus. Six rats were used for each dose of drug to be examined and control values for paw-flick and tail-flick were calculated by averaging the control responses for each group of 6 animals.

After intrathecal administration of the tachykinin antagonist(s), the paw-flick and tail-flick latencies were measured at 1, 3 and 5 minutes post-injection. The average paw-flick or tail-flick latencies for the 6 rats were calculated for each timepoint and were expressed as a percentage of the pre-drug control value .

In the carrageenan treated rats, the control values were estimated as described above, before the intraplantar injection of carrageenan. Four hours after the carrageenan was administered, the rats were re-tested for paw-flick and tail-flick latencies and the average value for each group expressed as a percentage of the control value. The drug was then administered intrathecally and the latencies tested at 1,3 and 5 minutes post-injection as described above.

The results for each group of 6 rats were meaned and expressed as a percentage of the total mean control value for all the rats being used in that particular experiment. This usually was from a total number of 24 rats. The graphs are shown in Figures 7.5, 7.6 and 7.7.

Figure 7.4

The glabrous paw-flick response

This photograph demonstrates the positioning used to hold the rat for the glabrous paw-flick response. The rat was held with its forelimbs raised, permitting a free range of movements in the rear quarters of the rat. The foot was placed over the light source in such a manner that the infrared beam was focused on the most padded part of the foot and thus presented minimal damage.

To assess statistical significance of changes between the vehicle and drug-induced response in each test, the students unpaired t-test was employed. When comparing changes between the non-carrageenan and carrageenan sensitised state, the statistical significance of changes was assessed by the Mann-Whitney U-test. This is a non-parametric statistical analysis test which makes no assumptions of a normal distribution of data and errors. Significance was accepted at probability values of $P \leq 0.05$.

7.4 RESULTS

7.4.1 Control Paw-flick And Tail-flick Responses.

After transportation from the animal storage room to the laboratory, the rats were always left for a minimum period of 30 minutes to enable them to adjust to the environment. There was free access to diet and water during this period. Three control responses to both tail-flick and paw-flick, in turn, were obtained for each rat and from these an average response time was calculated. To minimise sensitisation of the behavioural responses to the noxious heat stimulus, the paw-flick and tail-flick responses were alternated over the three control responses and tests repeated at not less than 2 minute intervals.

7.4.2 Nociceptive Testing in Non-Carrageenan treated Animals.

The effect of two tachykinin antagonists, L-659,874 and GR 82334 (highly selective for NK_2 and NK_1 receptors respectively) were quantified on nociceptive responses to the tail-flick and dorsal paw-flick tests. The tail-flick test has previously been widely used for determination of acute nociceptive thresholds (Cridland and Henry, 1986,1988; Rupniak *et al,* 1993), although there has been sparse and conflicting evidence for a role of tachykinins in mediating this response (Lembeck *et al,* 1981; Piercey *et al*, 1981; Yashpal *et al*, 1982; Gamse and Saria, 1986). The paw-flick test has also been widely used as a method of nociceptive testing, however most experimenters have relied heavily upon using the glabrous skin on the ventral surface of the foot. In electrophysiological experiments we found that this type of skin did not respond to cutaneous application of mustard oil, which is believed to specifically activate C fibre nociceptors (Lynn and Carpenter, 1982). As such polymodal C nociceptor afferents are very important in mediating nociceptive thermal response (Perl, 1984), it was decided to modify the paw-flick test by focusing the IR beam on the dorsal (hairy) surface of the foot;

this was achieved by gently bending the foot in such a way that a paw-flick could easily be achieved (see Figure 7.3).

(a) Effect of Intrathecal L-659,874.

Due to limits on both time and the number of animals available, it was decided to initially test only one high dose of the selective NK_2 antagonist; if a change in nociceptive latencies for tail-flick or paw-flick occurred then a dose-response curve could be completed. Six rats were intrathecally injected with L-659,874 and the tail-flick and dorsal paw-flick tests measured at 1,3 and 5 minutes post-injection as previously described. The results are graphically expressed in Figures 7.5 and 7.6 as a percentage of the DMSO control.

(ai) Tail-flick Test

At 1 minute post-intrathecal injection of 3nmol L-659,874, there was a very small, but insignificant decrease in the tail-flick latency to 88 ± 8% (mean ± s.e.m.) of the DMSO control ($P>0.05$, Unmatched Students t-Test). Similarly at the 3 and 5 minute test points, the 3nmol L-659,874 tail-flick latency was not significantly different to that of the DMSO control. However, as can be seen in Figure 7.5a, the intrathecal injection of DMSO gave an increase in Tail-flick latency. This may be due to either a vehicle effect or more likely, stress-induced analgesia in this group of 6 rats, so the experiment should be repeated under more closely controlled stress-free circumstances. When responses in the presence of L-659,874 were compared with the saline control, there was also no statistically significant difference in their effects.

(aii) Dorsal Paw-flick Test

As can be seen in Figure 7.6a, the 3nmol intrathecal injection of L-659,874 resulted in no statistical difference in dorsal paw-flick latency as compared to the DMSO vehicle control. At each of the time points tested 1,3 and 5 minutes, the dorsal paw-flick latency was 88 ± 9%; 87 ± 11% and 83 ± 10% (mean ± s.e.m.) of the vehicle control latencies respectively.

The result is surprising as it does not agree with previous electrophysiological studies on thermal nociception (see Chapters 2 and 4) or with behavioural experiments carried out by my colleague (Young *et al,* 1995).under similar (but not identical) circumstances to the present study. In the latter experiments it was found that a 3nmol intrathecal injection of L-659,874 caused a

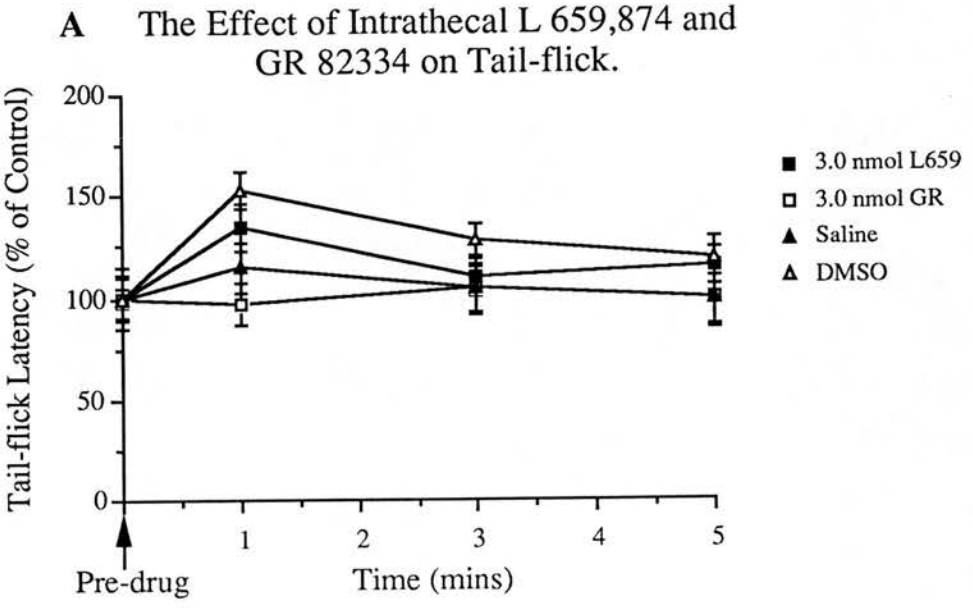
Figure 7.5

The effect of intrathecal tachykinin antagonists on the rat tail-flick response.

Tail-flick latency is plotted against time for the basal tail-flick latency and then at 1, 3, and 5 minutes post-intrathecal injection of the highly selective tachykinin antagonists L-659,874 or GR 82334.

(a) Demonstrates the effect of a 3 nmol intrathecal injection of either L-659,874 or GR 82334 on the rat tail-flick latency. Neither of the selective NK_1 or NK_2 antagonists showed any significant change to their respective saline or DMSO controls.

(b) Demonstrated the effect of a 0.1, 0.3 and 3nmol intrathecal co-injection of L-659,874 and GR 82334. The graph demonstrates that the dose-response curves to the two highest doses of intrathecal drug combination were not significantly different to that of the vehicle control at any of the time-points tested, however, at the lowest dose tested (0.1 nmol) there was a slight analgesic type action with a small but significantly decreased tail-flick from the vehicle control. This is perhaps a pro-nociceptive action of the drug combination at this dose. Values are the means ± s.e.m. from 6 separate determinations.


A The Effect of Intrathecal L 659,874 and GR 82334 on Tail-flick.
Tail-flick Latency (% of Control)
200
150
100
50
0
1
2
3
4
5
Pre-drug
Time (mins)
3.0 nmol L659
3.0 nmol GR
Saline
DMSO

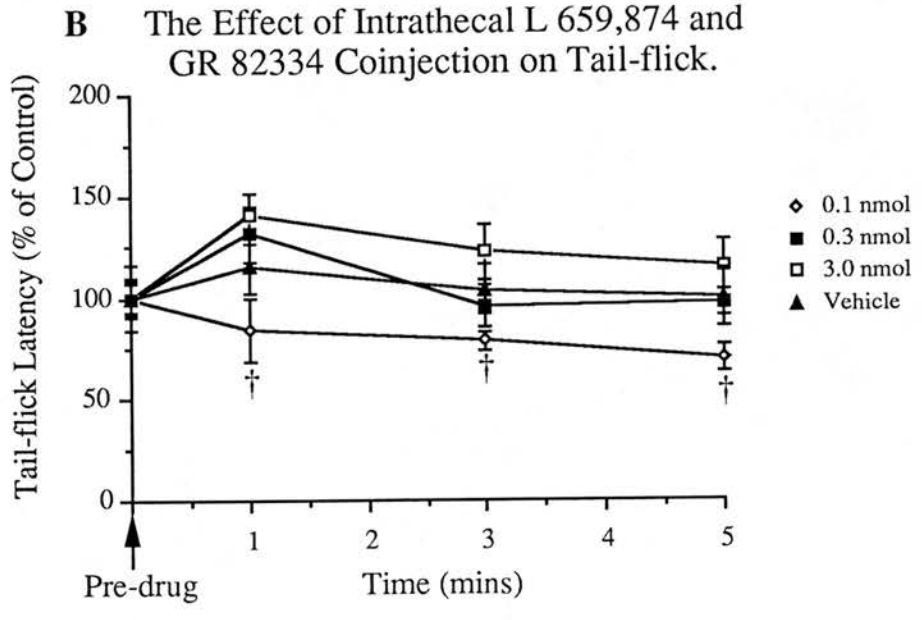

B The Effect of Intrathecal L 659,874 and GR 82334 Coinjection on Tail-flick.
Tail-flick Latency (% of Control)
200
150
100
50
0
1
2
3
4
5
Pre-drug
Time (mins)
0.1 nmol
0.3 nmol
3.0 nmol
Vehicle

Figure 7.6

The effect of intrathecal injection of tachykinin antagonists on the rat dorsal paw-flick response before and after 2% unilateral intraplantar carrageenan injection.

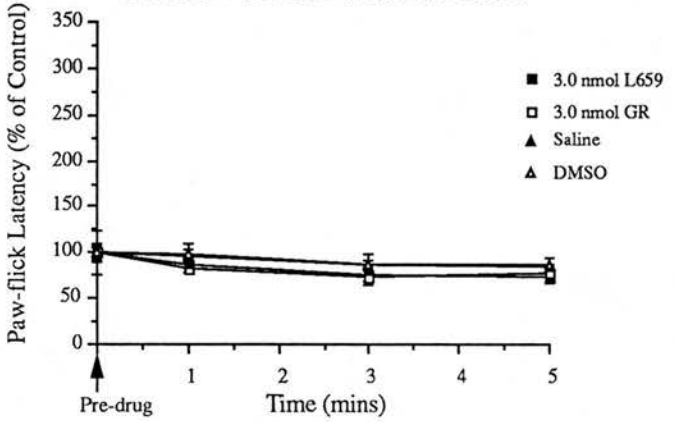
The effect of intrathecal injection of the highly selective NK_1 and NK_2 antagonists GR 82334 and L-659,874 on the dorsal paw-flick response were screened individually at a 3 nmol dose (a), followed by a co-injection of 0.1, 0.3, and 3.0 nmol of the combination. Four hours after carrageenan had been injected into the dorsal surface of the left hindpaw, the effect of the NK_1 and NK_2 antagonists on the dorsal were screened individually (c) and in combination (d).

Figure (a) demonstrates that a 3nmol injection of either L-659,874 or GR 82334 was not significantly different to the vehicle controls used (DMSO and saline for L-659,874 and GR 82334 respectively). However, 4 hours after a 2% carrageenan injection to the dorsal surface of the foot, an intrathecal injection of 0.1, 0.3 or 3.0 nmol L-659,874 caused a marked and highly significant increase in dorsal paw-flick latency in comparison to the DMSO control. (Figure (c)). A similar effect was seen after intrathecal injection of GR 82334, however only at the highest dose injected was there any significant increase in dorsal paw-flick latency compared to the saline control.

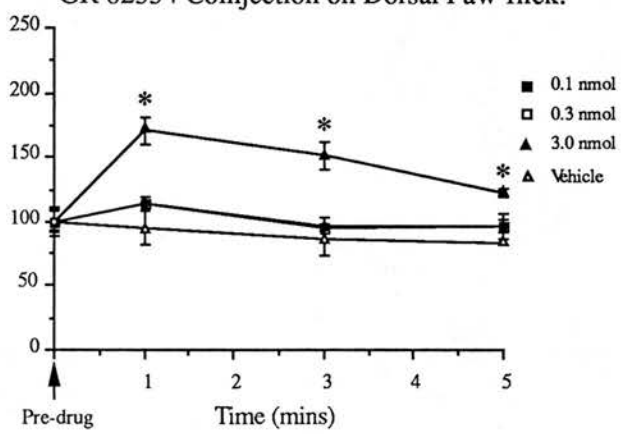
Figure (b) demonstrates the effect of an intrathecal co-injection of the two drugs under study on the dorsal paw-flick latency, it can be seen that the lowest dose of combination had no significant effect on the response whilst the two higher doses caused a dose-related increase in the paw-flick latency compared to the vehicle control. The effect of this drug-combination was not investigated in the carrageenan-sensitised model due to lack of resources available.

Normal

A

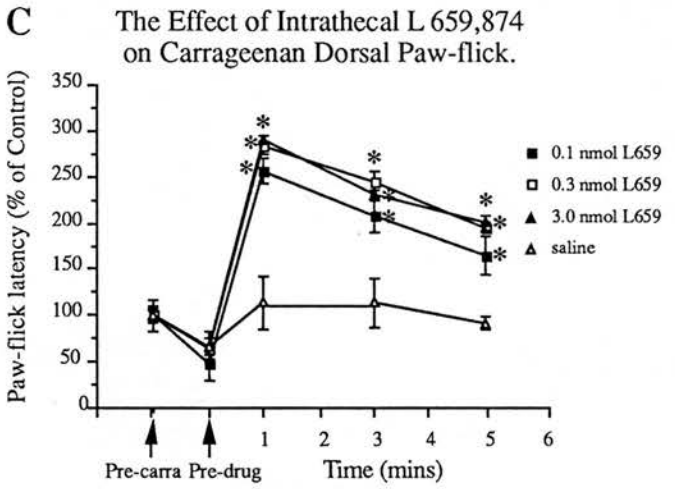


B



Carrageenan

C



D

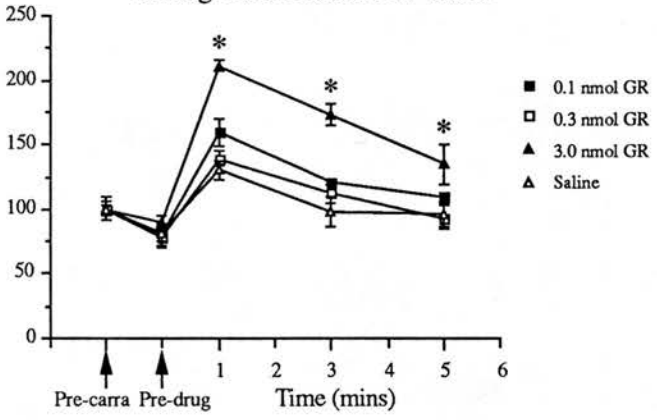
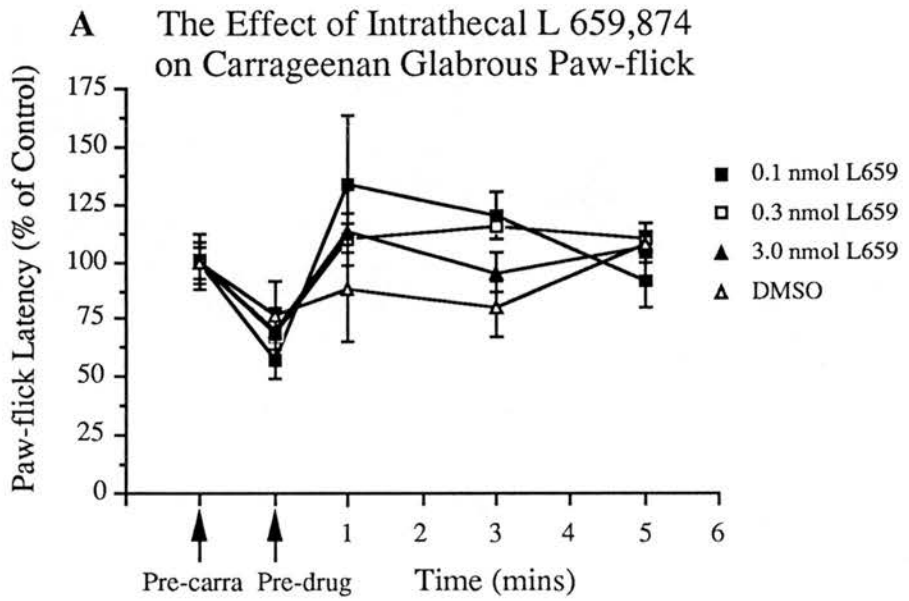
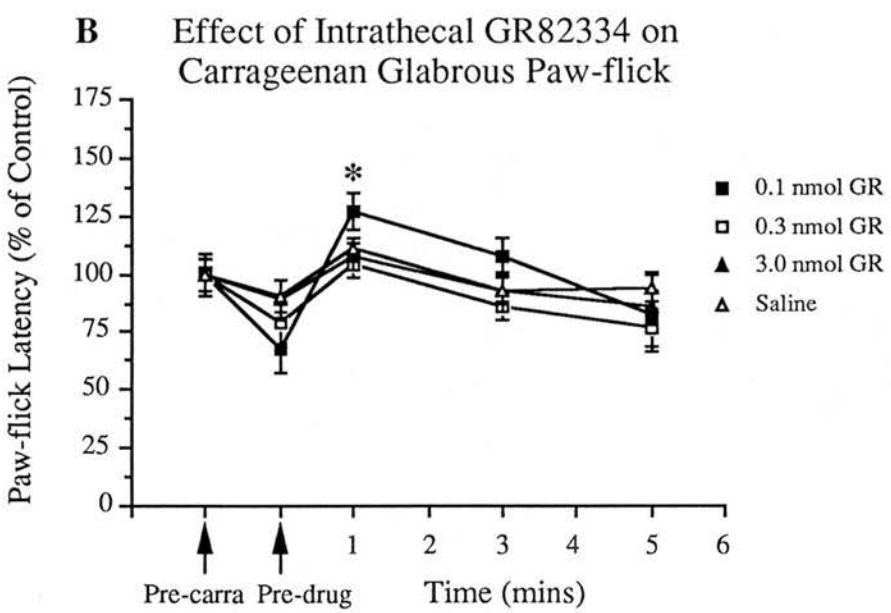


Figure 7.7

The effect of intrathecal injection of tachykinin antagonists on the rat glabrous paw-flick response before and after 2% unilateral intraplantar carrageenan injection.

The effect of intrathecal injection of the highly selective NK_1 and NK_2 antagonists GR 82334 and L-659,874 on the glabrous paw-flick response were screened four hours after carrageenan had been injected into the dorsal surface of the left hindpaw.Figures (a) and (b) demonstrate that at none of the doses tested (0.1, 0.3 and 3.0 nmol) did the highly selective NK_2 or NK_1 antagonists result in any significant change in carrageenan glabrous paw-flick latency. As these drugs were ineffective in the sensitised state (where it is more likely to see an effect) it was decided not to test these antagonists on the non-carrageenan glabrous paw-flick.


A
The Effect of Intrathecal L 659,874
on Carrageenan Glabrous Paw-flick
Paw-flick Latency (% of Control)
175
150
125
100
75
50
25
0
1
2
3
4
5
6
Pre-carra
Pre-drug
Time (mins)
0.1 nmol L659
0.3 nmol L659
3.0 nmol L659
DMSO


B
Effect of Intrathecal GR82334 on
Carrageenan Glabrous Paw-flick
Paw-flick Latency (% of Control)
175
150
125
100
75
50
25
0
*
1
2
3
4
5
6
Pre-carra
Pre-drug
Time (mins)
0.1 nmol GR
0.3 nmol GR
3.0 nmol GR
Saline

marked increase in paw-flick latency (to a maximal increase of 176% at 3 minutes post-intrathecal injection) compared to the DMSO control. One plausible explanation which can be offered is that the sample size used here (n=6) was much smaller than in the study conducted by colleagues. It seems quite possible that due to variable controls and the short time available for these studies to be carried out, the sample was not large enough to reflect a truly representative result. Another possibility is that variation occurred due to differing assessment methods and pre-treatment times in the dorsal paw-flick test. In my colleague's behavioural data, the rat's paw and limb were held flat over the IR source, whereas in this set of experiments the limb was slightly raised which resulted in a shorter average paw-flick latency.

(b) Effect of Intrathecal GR 82334.

In an analogous manner to tests for L-659,874, the highly selective NK_1 antagonist GR 82334 was intrathecally injected only at one dose before proceeding with tail-flick and dorsal paw-flick tests. A total of 12 rats were used, 6 for the drug under investigation and 6 for the saline controls; after intrathecal injection with L-659,874, the tail-flick and dorsal paw-flick tests were measured at 1,3 and 5 minutes post-injection as previously described. The results are graphically expressed in Figures 7.5 and 7.6 as a percentage of the saline control.

(bi) Tail-flick Test

It can be observed in Figure 7.5a that the 3nmol intrathecal injection of the highly selective NK_1 antagonist GR 82334, resulted in no significant change in tail-flick latencies at any of the time points tested, compared to the saline control. At 1, 3, and 5 minutes post-intrathecal injection, the tail-flick latencies were 96.2 ± 10%; 113 ± 12% and 109.5 ± 13% (mean ± s.e.m.) of the saline control respectively ($P>0.05$ Unmatched Students t-Test). At 3 and 5 minutes post-i.t. injection the curves for both GR 82334 and the saline control are closely matched.

(bii) Dorsal Paw-flick Test

There was no significant difference between the mean dorsal paw-flick latencies taken from 6 rats intrathecally injected with GR 82334 and a further 6, intrathecally injected with the vehicle control, saline. As can be seen in Figure 7.6a, at 1, 3, and 5 minutes post-intrathecal injection the paw-flick latencies for the GR 82334 treated rats were 86.2 ± 13.8%; 85.8 ± 15.0%; and 92.5 ± 10.6% of the

(c) Effect of Intrathecal Combination of L-659,874 and GR 82334

It was decided to investigate the possibility of a synergistic interaction between NK_1 and NK_2 receptors acting to produce a facilitated analgesic action. This was tested by giving a co-injection of NK_1 (GR 82334) and NK_2 (L-659,874) receptor antagonists. The effect of intrathecal application of the combination on dorsal paw-flick and tail-flick was tested in a total of 18 rats, with 6 in each dose group of 0.1nmol, 0.3nmol and 3nmol of the combined total drug concentrations; a further 6 animals were used to test the vehicle control combination (DMSO and saline). The results are graphically expressed in Figures 7.6 and 7.7 as a percentage of the control for each group. The baseline control values for each group of animals, whilst not statistically different from the mean control value, were a little variable. This can most probably be accounted for by the inexperience of animal handling over the short time available for this project (2 weeks).

(ci) Tail-flick Test

The response curve for the two higher doses of the NK_1/NK_2 combination (0.3 and 3nmol), followed the same pattern with a small, but insignificant increase in tail-flick latency at 1 minute post-intrathecal injection (to 115 ± 11% and 123 ± 15% of saline controls respectively, mean ± s.e.m.), which then fell towards baseline values for subsequent tests at 3 and 5 minutes (Figure 7.6b). As the vehicle also caused a similar increase in tail-flick latency at 1 minute post-intrathecal injection, this suggests this was either due to a vehicle-induced effect or to the stress of restraint and/ or injection and had no connection with any analgesic effect of the drugs.

The lower dose of the NK_1/NK_2 combination (0.1nmol) produced an entirely different response, showing a small, but significant decrease in tail-flick latency at 1 minute post-intrathecal injection (to 73 ± 14% of the saline control; mean ± s.e.m., $P<0.01$ Mann Whitney U-test). Similarly, at both the 3 and 5 minute time points post-intrathecal injection of the combined drugs, there was a significant decrease in the tail-flick response, in comparison to the vehicle control (75 ± 4% and 69 ± 6% respectively).

(cii) Dorsal Paw-flick Test

The vehicle control had no significant effect on paw-flick latencies compared to baseline. However, each of the three NK_1/NK_2 drug combination doses followed the same distinctive response pattern, with a sharp increase in paw

(cii) Dorsal Paw-flick Test

The vehicle control had no significant effect on paw-flick latencies compared to baseline. However, each of the three NK_1/NK_2 drug combination doses followed the same distinctive response pattern, with a sharp increase in paw withdrawal latency at 1 minute post-intrathecal injection, which gradually fell towards control values at the 3 and 5 minute time points (see Figure 7.6b).

The lowest dose (0.1nmol) of the NK_1/NK_2 antagonist combination slightly but insignificantly, raised the paw-flick latency above that of the vehicle control at 1, 3 and 5 minute intervals post-intrathecal injection to a maximum of 119 ± 6% at 1 minute post-intrathecal injection. Although there was no significant difference at any time-point tested, it did follow the general 'trend' of the higher doses. This result is most likely to be due to a short lasting action of the low dose of the drug combination, however, it could possibly be due to stress induced opioid release (Calcagnetti *et al*, 1990) as it was only 1 minute post-injection.

The two higher doses of the NK_1/NK_2 antagonist combination which were tested (0.3 and 3nmol), raised the paw-flick latency above that of the saline vehicle at all 3 time points tested (1,3 and 5 minutes post intrathecal injection) in a dose related manner. The highest dose of drug combination tested (3nmol), caused a marked and significantly raised dorsal paw-flick latency compared to the vehicle control. At 1 minute post-intrathecal injection the paw-flick latency was maximally increased to 178 ± 11% of vehicle control (mean ± s.e.m., $P<0.05$ Unmatched Students t-Test) and at 3 and 5 minutes post-intrathecal injection it was still markedly increased from the vehicle control to 175 ± 12% and 146 ± 4% of vehicle control respectively.

Thus from the above results it can be seen that a 3.0 nmol combination of L-659,874 and GR 82334 resulted in a significantly greater increase in dorsal paw-flick latency than either drug alone at the same dose, suggesting a synergistic interaction between the selective NK_1 and NK_2 antagonists, although it is possible, but not likely (considering a standard non co-operative form of dose-dependence) that an equivalent double dose of either drug may have achieved the same result.

7.4.3 Nociceptive Testing in Carrageenan Treated Animals

The effect of the NK_1 and NK_2 receptor antagonists, GR 82334 and L-659,874, on nociceptive responses to paw-flick latencies were investigated in rats unilaterally injected with 2% lamda carrageenan into the dorsal surface of the left hindpaw. As these experiments aimed to focus on the nociceptive responses before

and after tachykinin antagonists in a 'sensitised' state of carrageenan inflammation, the tail-flick responses were omitted and attention was focused on the dorsal and glabrous paw-flick responses.

In forty eight rats, the dorsal surface of the foot was injected with 100µl of 2% carrageenan, which spread to the toes and around the glabrous region of the foot. After 4 hours, thirty six rats were intrathecally injected with tachykinin antagonist (18 each of GR 82334 and L-659,874) and a further twelve injected with vehicle (6 each of DMSO and saline). The dorsal paw-flick was then tested and a comparison made with glabrous (the ventral surface of the foot traditionally tested) paw-flick. The glabrous paw-flick was screened on a sensitised response, because it was more likely to see an effect of drugs in a sensitised state (in view of previous results with hairy skin- see Chapter 3).

(a) Effect of Intrathecal L-659,874

The effect of intrathecal application of the NK_2 antagonist on both dorsal and glabrous paw-flick was investigated in a total of 18 rats, 6 for each dose of antagonist (0.1nmol, 0.3nmol and 3nmol) and also a further six were used to investigate the effect of vehicle in carrageenan-treated animals. The results are graphically expressed in Figures 7.6 and 7.7 as a percentage of the control for each group.

(ai) Dorsal Paw-flick Test

As can be seen in Figure 7.6b, 3 hours after carrageenan injection, the paw-flick latency for each group of rats decreased. Although this value was not statistically different to the pre-carrageenan control, the general trend was a decrease in latency consistent with the generation of a hyperalgesic state. The average response time was decreased from 100 ± 6% in the pre-carrageenan controls to 82 ± 7% in carrageenan injected animals

All three doses of L-659,874 produced a marked and highly significant increase in the paw-flick latency at all three time-points investigated. At 1 minute post-intrathecal injection, each dose of the NK_2 antagonist produced its maximum increase in dorsal paw withdrawal latency (to 225 ± 12%; 249 ± 9%; 255 ± 3% of vehicle control for 0.1nmol, 0.3nmol and 3nmol of L-659,874 respectively; mean ± s.e.m., $P<0.05$ Unmatched Students t-Test). At 3 and 5 minutes post-intrathecal injection of L-659,874, the paw-flick latencies were still large and significantly raised from the DMSO control. Interestingly, at each subsequent time point, there was a gradual decrease in the mean paw-flick latency compared to the previous

test. At 3 minutes post-intrathecal injection the paw-flick latencies for each dose (0.1, 0.3 and 3.0 nmol) were 157 ± 15%; 217 ± 9% and 204 ± 4% of vehicle control respectively, (mean ± s.e.m., $P<0.05$ Unmatched Students t-Test) and at 5 minutes post injection the Paw-flick latencies had changed to 181 ± 12%; 213 ± 6% and 220 ± 8% respectively (mean ± s.e.m., $P<0.05$ Unmatched Students t-Test).

(aii) Glabrous Paw-flick Test

As can be seen in Figure 7.7a, 3 hours after carrageenan injection, the glabrous paw-flick latency for each group of rats decreased in a similar manner to the dorsal paw-flick results. Although this value was not statistically different to the pre-carrageenan control, the general trend was a decrease in latency consistent with the generation of a hyperalgesic state. This implies that although the carrageenan injection had been given into the dorsal surface of the foot, the ventral side had been similarly affected with both dorsal and ventral paw-flick producing a similar decrease in paw withdrawal latency post-carrageenan. The average response time for glabrous paw-withdrawal was decreased from 100 ± 7% in the pre-carrageenan controls to 68 ± 11% in carrageenan injected animals.

However, despite the redness and swelling associated with the carrageenan injected hindlimb, suggesting a hyperalgesic state, L-659,874, at all 3 doses tested, was ineffective at producing any significant changes in the glabrous paw-flick latency compared to control DMSO values. At 1 minute post-intrathecal injection (where normally a maximum change is observed), the paw-flick latencies for each of 0.1, 0.3 and 3.0nmol intrathecal injections were 152 ± 33%; 125 ± 13% and 128 ± 4% (mean ± s.e.m.) of vehicle control respectively ($P>0.05$, Unmatched Students' t-Test). Although the general shape of each dose response curve follows a pattern typical of an analgesic-type response, at none of the doses tested was the response to intrathecal L-659,874 significantly different from the vehicle control curve. This is perhaps due to the large variability surrounding this test which is masking the significance of the glabrous paw-flick response to the NK_2 antagonist.

(b) Effect of Intrathecal GR 82334

The effect of intrathecal application of the NK_1 antagonist on both dorsal and glabrous paw-flick was investigated in a total of 18 rats, 6 for each dose of antagonist (0.1nmol, 0.3nmol and 3nmol) and also a further six were used to investigate the effect of vehicle in carrageenan-treated animals. The results are

graphically expressed in Figures 7.6d and 7.7b as a percentage of the control for each group.

(bi) Dorsal Paw-flick Test

As can be seen in Figure 7.6d, the dose response curve for saline and each dose of the NK_1 antagonist followed the same basic pattern with a decrease in paw-flick latency after 3 hours of carrageenan. Although this decrease in latency was not significant for any of the drugs or saline, it is consistent with the generation of a hyperalgesic state, the average response time was decreased from 100 ± 6% pre-carrageenan control to 82 ± 7% post-carrageenan control. At 1 minute after intrathecal injection, there is an increase in each dose response curve and although only the highest dose of 3nmol GR 82334 was significantly different from the saline vehicle control (161 ± 3%, mean ± s.e.m., $P<0.05$ Unmatched Students t-Test), the lower two doses obviously follow the same trend. At 3 and 5 minutes post-treatment, the dose response curves fell gradually back towards pre-carrageenan values with again only the 3nmol dose being significantly different from the saline control (176 ± 9% and 140 ± 16% respectively, mean ± s.e.m., $P<0.05$ Unmatched Students t-Test). The results are graphically expressed in Figures 7.6d as a percentage of the control for each group

(bii) Glabrous Paw-flick Test

Figure 7.7b demonstrates that 3 hours after carrageenan injection, the glabrous paw-flick latency for each group of rats decreased in a similar manner to the dorsal paw-flick results. Although this value was not statistically different to the pre-carrageenan control, the general trend was a significant decrease in paw-flick latency post-carrageenan, indicating that hyperalgesia has developed. This implies that although the carrageenan injection had been given into the dorsal surface of the foot, the ventral side had also been affected as there was a similar decrease in paw withdrawal latency post-carrageenan. The average response time for glabrous paw-withdrawal was decreased from 100 ± 7.6% in the pre-carrageenan controls to 81.1 ± 8.9% in carrageenan injected animals.

As can be clearly observed in Figure 7.7b intrathecal injection of the two lower doses of GR 82334 did not cause any significant change in the dose response curve as compared to saline control at any of the three time-points tested. The lowest dose of 0.1nmol GR 82334 caused a small but apparently significant increase in glabrous paw-flick latency at 1 minute post-intrathecal injection to 118 ± 7% of the vehicle control, with no significant effect at the 3 and 5 minute tests.

As this effect occurred at the lowest dose of drug tested with no significant changes at higher doses used, it suggests that the increase in paw-flick latency is not due to a drug-induced effect and is perhaps due to a stress-induced analgesia in this group of 6 rats. Another explanation is that the effect is vehicle-induced as the general dose-response pattern follows the same general shape as the two higher doses.

7.5 DISCUSSION

The experimental models of tail-flick (D'Amour and Smith, 1941) and glabrous paw-flick (Woolfe and MacDonald, 1944) employed here, have been previously used by various experimenters and provide reliable and reproducible methods with which to assess the effects of locally administered tachykinin receptor antagonists on the spinally-elicited behavioural paradigms of nociception (Cridland and Henry, 1986, 1988; Lecci *et al,* 1991; Garces *et al,* 1992; Picard *et al,* 1993; Yashpal *et al,* 1993). However, in these models, the paw-flick response has been tested using the thick hind-paw glabrous skin (Lecci *et al,* 1991; Garces *et al,* 1992), on which, paw-withdrawal latencies have been demonstrated to vary, depending on the precise location of the thermal stimuli applied to different regions of the glabrous skin (Doucette *et al,* 1987). Additionally, in previous studies (see Chapters 3 and 4), we have found that the C-fibre selective chemical irritant mustard oil is ineffective when painted onto the glabrous skin of the hindpaw, demonstrating that this skin-type is less sensitive. As such polymodal C nociceptive afferents are very important in mediating nociceptive thermal responses, the paw-flick method of nociceptive testing, described by Woolfe and MacDonald, (1944) was additionally modified such that the foot under examination was positioned to permit the thinner 'hairy skin' of the hindpaw to rest over the light beam in such a way that a paw-flick could easily be achieved.

The technique standardly used for intrathecal injection of compounds into small rats, that of Hylden and Wilcox, (1980), was practised using the selective NK_1 antagonist SPOMe, known to cause caudally-directed biting and scratching behaviour upon its successful intrathecal delivery (Gamse and Saria, 1986), and only when the experimenter was fully accomplished with this method, were the experiments initiated. Although it is possible in a few cases that the drug somehow did not did not reach the spinal cord, generally, puncture of the dura was reliably indicated by a flick of the tail.

The results presented here demonstrate that intrathecal administration of each of the selective NK_1 antagonist GR 82334 (Hagan *et al*, 1991) and the selective NK_2 antagonist L-659,874 (McKnight *et al*, 1988) failed to produce potent antinociceptive activity in both the acute analgesiometric tests of dorsal paw-flick and tail-flick. However, the results obtained with the selective NK_1 receptor antagonist in the acute nociceptive tests of paw-flick and tail-flick are not entirely surprising as mixed reports have been obtained by other groups following intrathecal administration of selective NK_1 agonists and antagonists. SP or selective NK_1 antagonists (i.t.) have been shown to evoke a classical behavioural response of compulsive biting, licking and scratching of the corresponding limb, believed to be indicative of discomfort or pain (Hylden and Wilcox, 1981; Piercey *et al*, 1981; Sweeney and Sawynok, 1986; Post and Folkers, 1985; Gamse and Saria, 1986; Papir-Kricheli *et al*, 1990; Laneuville *et al*, 1988; Lecci *et al*, 1991). However, the scratching response may not actually be pain-related, but may be a spinal convulsive state or drug-related side effect (Frenk *et al*, 1988). Indeed, although there are some reports that i.t administration of SP or an NK_1 receptor agonist can significantly reduce the threshold for eliciting a thermal tail-flick reflex (Picard *et al*, 1993; Cridland and Henry, 1986, 1988; Yashpal *et al*, 1993; Post and Folkers, 1985) or a paw-pressure test (Sweeney and Sawynok, 1986); others claim that i.t. SP plays no role in the behavioural responses to tail-flick and hot plate test in the mouse or rat (Gamse and Saria, 1986; Piercey *et al*, 1981) or that i.t. SP can cause hypoalgesia (Doi and Jurna, 1981). Similarly, mixed effects have been described following i.t. selective NK_1 antagonists. Behavioural analgesia in the hot plate and tail-flick tests has been obtained with selective NK_1 antagonists (usually in relatively high doses), including some hexapeptide analogues of SP (i.t.) (Lembeck *et al*, 1981; Post and Folkers *et al*, 1985) or the non-peptide antagonist CP-96,345 (i.t. or i.p.) (Lecci *et al*, 1991; Picard *et al*, 1993; Yashpal *et al*, 1993). However, others have found that CP-96,345 or the more recently developed NK_1 antagonist CP-99,994 were ineffective in tail-flick, paw-flick or paw pinch tests (Elliott *et al*, 1992; Garces *et al*, 1992) as well as SP-mediated biting and scratching (Garces *et al*, 1992). Further, i.t. RP 67580 (which binds with higher affinity to the rat NK_1 receptor (Garrett *et al*, 1991)) was inactive in rat paw-pressure tests (Rupniak *et al*, 1993).

In addition, the results obtained behaviourally with NK_1 antagonists are in agreement with the electrophysiology experiments described in Chapter 2 which provided no clear evidence that NK_1 receptors participate in transducing brief nociceptive inputs to laminae III-V neurons of the rat spinal dorsal horn. In

accord, others have reported a similar lack of involvement of NK_1 receptors in mediating brief nociceptive inputs (both thermal and mechanical) to deeper dorsal horn neurons (Duggan *et al*, 1979; Willcockson *et al*, 1984; Fleetwood-Walker *et al*, 1990). However, again, there are mixed results with reports that i.t. administration of the highly selective non-peptide NK_1 antagonist CP-96,345 resulted in inhibition of responses of nociceptive dorsal horn neurons to C fibre electrical stimuli of the after discharge response to brief noxious pinch and heat (De Koninck and Henry, 1991; Radhakrishnan and Henry, 1991) with little effect on the initial phase of excitatory responses to brief heat stimuli and inconsistent effects on pinch-evoked excitation. It is possible that CP-96,345 is effective only on late components of nociceptive responses or of more sustained pain states, this is supported by the observation that CP-96,345 was able to significantly reduce the ventral root potentials evoked by C-fibre strength electrical stimulation in *in vitro* hemisected spinal cords, isolated from rat pups with ultraviolet light-induced thermal and mechanical hyperalgesia but not in naive, untreated rat spinal cords (Thompson *et al*, 1993).

The results obtained following i.t. administration of the selective NK_2 receptor antagonist L-659,874 in the acute nociceptive tests were unexpected. Although far fewer behavioural studies have been carried out for NKA and NK_2 receptors than for NK_1 receptors, the evidence to date suggests that NK_2 receptors are extremely important in mediating thermal nociceptive transmission in the spinal cord. Intrathecal NKA or selective NK_2 agonist results in caudally directed biting and scratching behaviour (Gamse and Saria, 1986) in addition to dose-dependent, transient decreases in the tail-flick and paw-flick response latencies to a noxious thermal stimulus (Cridland and Henry, 1986; Laneuville *et al*, 1988; Fleetwood-Walker *et al*, 1990; Picard *et al*, 1993), reversed following i.t injection of selective NK_2 antagonists (Fleetwood-Walker *et al*, 1990; Picard *et al*, 1993). Moreover, in electrophysiological studies we have demonstrated a role for NK_2 receptors in mediating acute nociceptive thermal (but not mechanical or innocuous cutaneous sensory responses) following administration of selective NK_2 agonists and antagonists (see Chapters 2 and 4). In agreement, other studies have demonstrated an important role for NKA and NK_2 receptors in mediating acute nociceptive responses: intracellular recording studies demonstrate that NKA could elicit a selective depolarisation in capsaicin-sensitive C fibres (Nagy *et al*, 1993) and further that the selective NK_2 receptor antagonist MEN 10376 inhibited ventral root potentials evoked by C fibre strength electrical stimulation of ipsilateral dorsal roots (Thompson *et al*, 1993). Noxious mechanical and noxious

thermal stimuli both increase the levels of immunoreactive NKA throughout the spinal cord (Duggan *et al*, 1990; Hope *et al*, 1990) and a role for NK_2 receptors in facilitating the nociceptive spinal flexor reflex has been suggested (Xu *et al*, 1991; Xu and Wiesenfeld-Hallin, 1992; Wiesenfeld-Hallin *et al*, 1994).

The reason for the spurious NK_2 results is perhaps due to the small number (6) of animals in each dose group used; this in combination with the examiner's inexperience in animal handling conceivably masked any true response to the selective NK_2 antagonist. In addition, although L-659,874 shows extremely good selectivity for NK_2 over NK_1 and NK_3 receptors (McKnight *et al*, 1988; Williams *et al*, 1988), the possibility of unidentified side effects playing a role can not be ruled out. However, it may be that not all the injections actually reached the spinal cord (although a tail-flick was generally observed, an indication that the dura had been ruptured) or that inadequate concentrations were reached to block the behavioural nociceptive tests *in vivo*.

There is good evidence that the tachykinins SP and NKA are synthesised together and co-exist in primary afferents (Carter and Krause, 1990; Dalsgaard *et al*, 1985; Helke *et al*, 1990). Although different patterns of stimuli have been demonstrated to release each tachykinin (see Duggan *et al*, 1987,1988,1990; Go and Yaksh, 1987; Kuraishi *et al*, 1989; Hope *et al*, 1990; Lang *et al*, 1991), it is possible that the action of each tachykinin can potentiate the action of the other in order to jointly mediate the responses to acute nociceptive stimuli. Therefore the behavioural nociceptive thresholds to the tail-flick and paw-flick tests were measured following an i.t. co-injection of the selective NK_1 and NK_2 antagonists, GR 82334 and L-659,874 respectively.

The results demonstrate that following the two highest doses of i.t. drug combination used (0.3 and 3 nmol), there was a small but insignificant increase in tail-flick latency at 1 minute post-i.t. injection. However, as the vehicle also caused a similar increase in tail-flick latency at 1 minute post-i.t. injection, this suggests that this was due to a vehicle-induced effect or to the stress of restraint (Calcagnetti *et al*, 1990) and/or injection and had no connection with any analgesic effect of the drugs. Surprisingly however, the lowest dose of the drug combination used (0.1 nmol) produced an entirely different response, showing a small but significant decrease in tail-flick latency at 1, 3 and 5 minute post-intrathecal injection in comparison to the vehicle control. It is possible that some distinct and still authentic drug effect is most apparent at lower doses if observed by side effects at higher doses. However, again in this series of experiments the

numbers of rats in each dose group was low and therefore before any conclusions can be drawn this experiment should be repeated using a larger sample.

In contrast to the general lack of effect on the tail-flick response, following i.t. combination of the NK_1/NK_2 antagonists GR 82334 and L-659,874, there was a dose-related increase in dorsal paw-flick latency at each of the time-points tested. The two higher doses of combination used (0.3 and 3 nmol) significantly raised the paw-flick latency above that of the saline vehicle, the highest dose causing a marked and significant augmentation. Although at the lowest dose of antagonist combination used (0.1 nmol) this increase was insignificant, it did follow the general trend of the two higher doses, suggesting a short-lasting action of the low dose. These results demonstrate that although a singular i.t. injection of either the selective NK_1 or the NK_2 antagonist used (GR 82334 and L-659,874) has no significant effect on the dorsal paw-flick, when they are administered as a co-injection (at the same dose) there is a dose-dependent increase in the paw-flick latency. A plausible explanation for these results is that the highest singular dose of either NK_1 or NK_2 antagonist used was subliminal to elicit any significant effect on the behavioural response to dorsal paw-flick, however in combination a synergistic effect is produced, sufficient to produce a significant attenuation of the behavioural response. Although we have not investigated a possible synergy between NK_1 and NK_2 receptors on acute electrophysiological responses, in the model of sustained C fibre activity following cutaneous mustard oil, one neuron was tested with a combination of both NK_1 and NK_2 antagonists. Whereas singularly, each antagonist only caused a small inhibition of the mustard oil-induced firing, when ionophoresed in combination, a marked and long-lasting inhibition of the sustained C-fibre activity was produced. Although this model is not directly comparable with the behavioural paradigm of dorsal paw-flick, it does demonstrate the capability of NK_1 and NK_2 receptors to act in synergy.

The apparent lack of conjoint involvement of NK_1 and NK_2 co-injection in the tail-flick response can conceivably be explained by the differences in the tail skin-type compared to the hairy skin of the paw. The glabrous, thick skin on the tail is likely to contain different proportions of fibre types (and therefore different combinations of neuropeptides) compared to the thinner hairy skin of the foot (Treede *et al*, 1992). This is emphasised by the lack of effect of the C-fibre selectant algogen, mustard oil, which results in an increased background activity only on the hairy and not the plantar (glabrous) skin of the hindpaw (see Chapters

The carrageenan-induced inflammation model used here was modified from a protocol characterised by others (Iadarola *et al*, 1988; Draisci and Iadarola, 1989). Rather than the intraplantar injection previously practised, inflammation was induced in the dorsal (hairy) surface of the hindpaw with a single injection of 2% lambda (λ) carrageenan. When inflammation had fully developed, both glabrous and dorsal paw-flick responses were measured, although the latencies were not statistically different from the pre-carrageenan controls, the general trend was a decrease in glabrous as well as dorsal paw-flick latencies consistent with the generation of a hyperalgesic state.

Despite the redness and swelling associated with the carrageenan-injected limb, suggestive of development of inflammation which may result in a hyperalgesic state, L-659,874 (at all 3 doses tested) was ineffective at producing any significant changes in the glabrous paw-flick latency compared to control DMSO values. Although this latter result was initially surprising, on close consideration, it can be seen that even although a 4 hour period was allowed following carrageenan injection, neither of the observed decreases in glabrous or the dorsal paw-flick were significant compared to pre-carrageenan controls. Therefore, if inflammation had not fully developed at the site of carrageenan injection (on the hairy surface), it is very unlikely that the glabrous side of the paw would be sensitised sufficiently to observe any changes in paw-flick compared to the acute state. This experiment should be repeated with local injections of 2% λ-carrageenan to both the dorsal and the glabrous surfaces of the hindpaw, left for longer periods for inflammation to develop.

In contrast, following carrageenan injection, intrathecal administration of each of 3 doses of L-659,874 used, produced a marked and highly significant increase in the dorsal paw-flick latency at all three time points investigated, even at 5 minutes post-i.t. injection, the paw-flick latencies were still large and significantly raised from the DMSO control. Although as previously mentioned, the carrageenan-induced inflammation had not fully developed, there obviously was a greater level of sensitisation compared to the glabrous skin accounting for the marked response following i.t. L-659,874.

Although as yet there are no reports of the behavioural responses mediated by NK_2 receptors in sustained nociceptive states these results are in agreement with electrophysiological models of sustained or chronic pain associated with inflammation. Sustained activation of lamina IV/V neurons, induced by repeated peripheral application of the selective C fibre afferent stimulant, mustard oil (over 10-20 minutes) could be inhibited by ionophoretic

induced by repeated peripheral application of the selective C fibre afferent stimulant, mustard oil (over 10-20 minutes) could be inhibited by ionophoretic application of L-659,874 around these neurons (see Chapter 4). The flexor reflex facilitation evoked by damaging cutaneous thermal stimuli (80°C for 0.5 seconds) was significantly potentiated by NKA (i.t.) (Xu and Wiesenfeld-Hallin, 1992). Likewise, MEN 10207, a selective NK_2 receptor antagonist, effectively blocked the prolonged facilitation of the flexor reflex (lasting for up to 1 hour) that had been induced by C-fibre strength conditioning stimulation of the gastrocnemius-muscle nerve (Xu *et al*, 1991). All these experiments provide evidence that NK_2 receptors are indeed important in more prolonged nociception.

Further, in this carrageenan model, there was generally no significant effects in the glabrous paw-flick test (compared to the saline control) following i.t administration of GR 82334. As the carrageenan-induced inflammation was not fully developed (even on the dorsal surface of the paw) it is not surprising that following i.t. injection of GR 82334, there was no change in glabrous paw-flick latency compared to acute studies. However, only the highest dose of the selective NK_1 antagonist GR 82334 (i.t.) used (3 nmol) produced a marked and highly significant increase in the dorsal paw-flick latency at all three time points investigated, which is surprising as previously we have demonstrated that in a mustard oil-induced model of sensitisation, GR 82334 is highly potent at antagonising the facilitated sensory responses to both noxious thermal and innocuous stimuli (see Chapter 4) at relatively low ejection currents. Possible explanations are firstly that SP-LI is not detectable in the C polymodal nociceptor units found in the hairy skin (Lawson *et al*, 1994) but is found in A fibre high threshold afferents (Lawson *et al*, 1994). In acute nociceptive testing therefore, the involvement of SP may be minimal, depending on the properties and location of the noxious stimulus. However following the hyperalgesia and sensitisation produced by the inflammatory agent carrageenan, central sensitisation occurs which causes activity-dependent alterations in dorsal horn neurons such that they begin to respond in an abnormal or exaggerated way to Aδ (and perhaps Aβ) and C afferent inputs (Woolf, 1991). SP is therefore likely to be released in such an inflammatory state, however, as described earlier, the carrageenan-induced inflammation had apparently not fully developed and therefore the release of SP would be minimal and only at the highest dose of NK_1 antagonist administered was there likely to be any effect.

In support of this reasoning, previous behavioural models of hyperalgesia and inflammation have suggested a role for SP and NK_1 receptors in sensitised

models of pain (Garret *et al,* 1991; Birch *et al,* 1992; Lembeck *et al,* 1992; Nagahisa *et al,* 1992; Chapman and Dickenson, 1993; Yashpal *et al,* 1993) and although spurious non-NK_1 receptor mediated effects may have been involved with these compounds (Schmidt *et al,* 1992; Nagahisa *et al,* 1992; Chapman and Dickenson, 1993; Rupniak *et al,* 1993), this suggests a role for NK_1 receptors in these hyperalgesic models. In addition, hyperalgesic and inflammatory models have been shown to increase the biosynthesis of SP-IR, PPT-1 mRNA and NK_1 binding sites in the spinal dorsal horn and dorsal root ganglia of rats (Minami *et al,* 1989; Donaldson *et al,* 1992; Noguchi *et al,* 1992; Schafer *et al,* 1993; Stucky *et al,* 1993; McCarson and Krause, 1994) and increase the release of SP-IR into the spinal cord (Go and Yaksh, 1987; Oku *et al,* 1987; Kuraishi *et al,* 1989; Schaible *et al,* 1990; McCarson and Goldstein, 1991; Garry and Hargreaves, 1992).

These results highlight the importance of the tachykinins SP and NKA (and their respective NK_1 and NK_2 receptors) in the involvement of both acute and sustained nociceptive transmission. As this was a relatively short (3 week) project and the numbers of weanling rats available were limited, each dose group was small, therefore to overcome any unnecessary variability, a larger study should be completed. In addition, the inflammation produced 4 hours following carrageenan injection was apparently not fully developed and this series of experiments should therefore be repeated over a longer time scale.

CHAPTER 8:
Conclusions

This project has used a number of different techniques to investigate the role of the tachykinin neuropeptides SP and NKA, their respective NK_1 and NK_2 receptor subtypes and some intracellular mediators in both acute and sustained nociceptive inputs to dorsal horn neurons. Electrophysiological extracellular recordings from multireceptive laminae III-V dorsal horn neurons were combined with ionophoresis of selective drugs to address the relative role of NK_1 and NK_2 receptors firstly in brief nociceptive somatosensory prosessing in the dorsal horn, in addition to the sustained neuronal activity induced by repeated cutaneous application of the C-fibre selective chemical algogen mustard oil. As the intracellular mechanisms involved in the sensitisation of spinal dorsal horn neurons brought about by such sustained nociceptive inputs are unknown, subsequent electrophysiological recordings and biochemical activation experiments were carried out to assess whether protein kinase C (PKC) plays a significant role in sustained neuronal activation by mustard oil and further to examine sensitivity of the latter to NK_2 receptor antagonists. Additional studies were carried out using the characteristic expression of mustard oil-induced c-fos mRNA within the superficial dorsal horn as a marker to further assess the possible tachykinin involvement in this model of sustained C-fibre activity. Finally, the effects of intrathecal injection of tachykinin antagonists were assessed on behavioural paradigms of nociception before and after unilateral carrageenan-induced inflammation.

The results presented here provided no clear evidence that NK_1 receptors participate in transducing brief nociceptive inputs to the rat spinal cord in either behavioural or electrophysiological models. In contrast, in the latter, when NK_1 receptor antagonists were ionophoretically applied around the cell, they caused a modest facilitation of their responses to innocuous brush, suggesting that their lack of effect on nociceptive responses was not due to failure to reach relevant sites at effective concentrations. These results are entirely in agreement with previous studies using cats (Fleetwood-Walker *et al*, 1987, 1990). However other *in vivo* electrophysiology studies have provided very mixed results following ionophoresis of selective NK_1 agonists and antagonists. Some groups reporting excitation (Henry, 1976; Randic and Miletic, 1977; Sastry, 1979; Zieglgansberger and

Tulloch, 1979; Piercey *et al*, 1981; Kellstein *et al*, 1990; Hill *et al*, 1985; Salter and Henry, 1991) but others reporting complex mixed excitatory and inhibitory effects (Davies and Dray, 1980; Murase and Randic, 1984; Willcockson *et al*, 1984; Ryall and Pini, 1987) suggesting multiple sites or components of action. Similarly, mixed reports have been obtained from behavioural studies, some reports indicating that i.t. administration of selective NK_1 agonists and antagonists results in dose-dependent nociceptive/ antinociceptive syndromes respectively (Post and Folkers, 1985; Gamse and Saria, 1986; Sweeney and Sawynok, 1986; Lecci *et al*, 1991; Picard *et al*, 1993; Yashpal *et al*, 1993), whereas others have reported that SP plays no role in behavioural responses to nociception (Piercey *et al*, 1981; Gamse and Saria, 1986; Garces *et al*, 1992; Rupniak *et al*, 1993). Therefore it is apparent that although SP may play a minor role in transmission of brief nociceptive responses, it is not likely to be the main transmitter involved. In the sensitised/ inflamed state however, the actions of SP are clearly different.

The present results clearly demonstrate that following either prolonged exposure to mustard oil or unilateral intraplantar injection of the inflammatory agent carrageenan, NK_1 receptors played an important role in the transmission of both nociceptive and non-nociceptive responses, in agreement with others who found that NK_1 receptor antagonists failed to alter behavioural hyperalgesia induced in normal rats, but were highly effective analgesic agents in a variety of behavioural models of sustained/inflammatory pain (Garret *et al*, 1991; Yamamoto and Yaksh, 1991; Murray *et al*, 1991; Birch *et al*, 1992; Chapman and Dickenson, 1993; Yashpal *et al*, 1993). Likewise, others found NK_1 receptors could not be implicated in normal, baseline nociceptive processing, but were important in sustained nociceptive states involving damage and central sensitisation (Wiesenfeld-Hallin *et al*, 1990; Xu *et al*, 1992; Laird *et al*, 1993; Thompson *et al*, 1993). Similarly, although whilst SP was not released by noxious thermal stimuli at non-damaging levels (Kuraishi *et al*, 1989), both increasing the temperature to elicit chronic inflammatory damage and induction of experimental arthritis with intra-articular algogens do result in release of SP into dorsal horn (Duggan *et al*, 1988; Schaible *et al*, 1990), further suggesting that SP and NK_1 receptors are important following prolonged noxious stimulation. Inflammatory models of formalin and CFA have been shown to increase to biosynthesis of SP-IR, PPT-1 mRNA, NK_1 receptor mRNA and corresponding NK_1 binding sites suggesting that biosynthesis of SP in the spinal cord and primary sensory neurons can be increased by a sustained and inflammatory pain state. One possible mechanism could be a decrease in the rapid degradation of SP which normally occurs in the

spinal cord. Indeed, SP is normally released into a focal region of the superficial dorsal horn (Duggan *et al*, 1987, 1988) but spreads in the presence of peptidase inhibitors (Duggan *et al*, 1992). The endogenous neuropeptide CGRP has been postulated to have attenuate SP degradation, like peptidase inhibitors (le Graves *et al*, 1985), indeed CGRP co-localises with SP (Carr and Nagy, 1993; Levine *et al*, 1993) and its biosynthesis is upregulated during sustained nociceptive conditions Weihe *et al*, 1988; Schaible *et al*, 1994). In addition, CGRP has been shown to be co-released with SP during carrageenan-induced inflammation (Garry and Hargreaves, 1992). However, it is not yet clear whether the role in sensitisation of NK_1 receptors that emerges after inflammation is simply due to extra production, output and spread of SP to NK_1 sites some way from the release site or whether the receptors have in addition modified cellular responses to further inputs.

The present results demonstrate that in each of the models used, NK_2 receptors and possibly NKA are important mediators of both brief noxious thermal responses in addition to mustard oil-induced or carrageenan-induced sustained nociceptive activity. This is in agreement with a number of other studies which have demonstrated a role for NKA (and NK_2 receptors) in both acute and sustained nociceptive states (Fleetwood-Walker *et al*, 1987, 1990; Cridland and Henry, 1986; Xu *et al*, 1991; Urban *et al*, 1992; Xu and Wiesenfeld-Hallin, 1992; Nagy *et al*, 1993; Laird *et al*, 1993; Picard *et al*, 1993; Thompson *et al*, 1993).

The differences obtained between the results for NK_1 and NK_2 receptors are at first sight are surprising as there is good evidence that the tachykinins SP and NKA are synthesised together and co-exist in primary afferents (Dalsgaard *et al*, 1985; Carter and Krause, 1990; Helke *et al*, 1990). However, it is evident that SP and NKA have very different release (or degradation) patterns and roles in the spinal cord, both noxious (but not damaging) thermal and mechanical stimuli being effective stimuli to produce a diffuse and long-lasting release of NKA in the spinal cord (Duggan *et al*, 1990; Hope *et al*, 1990). It may be possible that CGRP, or another endogenous neuropeptide is tonically released in conjunction with NKA to limit degredation.

There is also a strong body of evidence suggesting that tachykinins and excitatory amino acids (EAAs) can act synergistically to facilitate nociceptive processing. Neuropeptides and EAAs are found to be co-localised in the terminals of C and some Aδ primary afferent fibres (De Biasi and Rustioni, 1988) therefore upon release these transmitters could act either pre- or postsynaptically. Both presynaptic and postsynaptic actions of neuropeptides on EAA transmission are

likely, since SP, neurokinin A or CGRP have been found to enhance the release of glutamate and aspartate from spinal cord dorsal horn (Kangraga and Randic, 1990), while others have found that both SP and NKA could modulate NMDA-receptor activity in acutely isolated dorsal horn cells (Willcockson *et al*, 1984; Dougherty and Willis, 1992; Rusin *et al*, 1992; Urban *et al*, 1994). In dorsal horn cells, membrane currents elicited by NMDA and quisqualate were predominantly enhanced by SP and NKA, whereas responses to kainate were unaffected by either of the neurokinins (Urban *et al*, 1994). However, in experiments with whole spinal cords, in the *in vitro* neonatal spinal cord with attatched tail preparation, these results could be reproduced only with NKA (Urban *et al*, 1994). When SP was replaced by the metabolically stable SPOMe or when SP was applied in presence of endopeptidase inhibitors, both NMDA and quisqualate responses were enhanced (Urban *et al*, 1994). The potent effect of antagonists of NK_1 and NK_2 receptors in blocking the enhancement of NMDA responses reinforces the view for a prominent role for NK receptors in the enhancement of NMDA-receptor responses (Urban *et al*, 1994; Rusin *et al*, 1992).

SP and NMDA interact in an apparently synergistic manner to activate dorsal horn neurons and facilitate their responses to non-noxious and noxious mechanical stimuli (Dougherty and Willis, 1991; Rusin *et al*, 1992; Song and Zhao, 1994), in potentiation of the nociceptive flexor reflex (Xu *et al*, 1992) and in behavioural analgesia (Woolf and Thompson, 1991; Mjellem-Joly *et al*, 1992). Conditioning by stimulation of C fibres or by application of SP facilitates NMDA- and quisqualate-receptor activation (Rusin *et al*, 1992; Urban *et al*, 1994; Dougherty and Willis, 1991) and the interaction can be blocked by various selective and non-selective NK-receptor antagonists (Rusin *et al*, 1992; Urban *et al*, 1994).

Therefore a selective synergy between NK_1/NK_2 and EAA receptors may have an important role in hyperalgesia by maintaining spinal hyperexciteability through the enhancement of NMDA receptor activity. It has been proposed that this interaction between NK and EAA receptors involves protein kinase C activation (Urban *et al*, 1994) as in both isolated cells (Rusin *et al*, 1992) and whole spinal cord (Urban *et al*, 1994), it was found that staurosporine blocked the interaction between the NK and EAA receptors but not the responses elicited by the application of NMDA alone. As staurosporine is a non-selective inhibitor of protein kinase C, other protein kinases may potentially also be involved, however, in isolated dorsal horn cells phorbol-12,13-dibutyrate (PDBu), a selective PKC activator, mimicked the effects of neurokinins on the NMDA-induced currents

(Rusin *et al*, 1992), further strengthening the hypothesis. Indeed, NK_1 and NK_2 receptors act through phosphoinositide hydrolysis, calcium mobilisation and stimulation of PKC. The activated PKC phosphorylates cytosolic and membrane proteins that might include the NMDA receptor and therefore offers a mechanism of NMDA-related synaptic transmission (Gerber *et al*, 1989). A molecular mechanism whereby PKC activation could promote the function of NMDA receptor channels has been described, where receptor phosphorylation results in diminished Mg^{2+} block and increased conductance at a given membrane potential (Chen and Huang, 1992). Clearly neurokinin receptors could initiate such a mechanism, perhaps underpinning the synergistic contribution they make with NMDA receptors to dorsal horn sensitisation.

Although there is compelling evidence for synergism between NK_1/NK_2 receptors and for the NMDA receptor operating through PKC, other experiments have shown that depolarisations of ventral roots, elicited by NKA, were completely and reversibly blocked by staurosporine whereas SP responses were unaffected (Urban *et al*, 1994). This suggests that other second messenger systems may be more important than PKC in the NK_1 receptor-, but not the NK_2 receptor-enhancement of NMDA receptors. In this project, selective NK_2 receptor antagonists prevented the mustard oil-induced PDBu translocation from cytosolic to membrane sites, consistent with the possibility that in this model (which results in hyperalgesia), NK_2 receptors may be acting in a PKC-dependent mechanism to promote NMDA receptor responsiveness. Other mechanisms are of course possible, PKC and other post-transducers of nociception and sensitisation have been relatively little explored, but potentially represent very important targets for new analgesics.

BIBLIOGRAPHY

Abbadie, C. and Besson, J-M. (1993). C-fos expression in rat lumbar spinal cord following peripheral stimulation in adjuvant-induced arthritic and normal rats. Brain Res. 607: 195-204.

Adrian, E.D. (1931). The messages in sensory nerve fibres and their interpretation. Proceedings of the Royal Society Series B 109: 1-18.

Adrian, E.D. and Zotterman, Y. (1926). The impulses produced by sensory nerve endings: Part 1. J. Physiol. 61: 49-72.

Advenier, C., Rouissi, N., Nguyen, Q.T., Emonds-Alt, X., Breliere, J.C., Neliat, G., Naline, E. and Regoli, D. (1992). Neurokinin A (NK-2) receptor revisited with SR 48968 a potent non-peptide antagonist. Biochem. Biophys. Res. Commun. 184: 1418-1424.

Akerman, B., Rosell, S. and Folkers, K. (1982). Intrathecal [D-Pro^{2},D-Trp^{7,9}]SP elicits hypoalgesia and motor blockade in the rat and antagonises noxious responses induced by substance P. Acta. Physiol. Scand. 114: 631.

Akers, R.F., Lovinger, D.M., Colley, P.A., Linden, D.J., Routtenberg, A. (1986). Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. Science 231: 587-589.

Alkon, D.L., Kubota, M., Neary, J.T., Naito, S., Coulter, D. and Rasmussen, H. (1986). C-Kinase activation prolongs Ca^{2+} dependent inactivation of K^{+} channels. Biochem. Biophys. Res. Commun. 134: 1245-1253.

Allam, D., Girdlestone, D. and Garret, C. (1992). Effects of the substance P antagonist, RP 67580, on neuronal activity in the locus coerulus. Br. J. Pharmacol. Proc. Suppl. 107: 227P.

Andres, K.H. and During, M. Von. (1973). Morphology of cutaneous receptors. In: Handbook of Sensory Physiology Vol. II Somatosensory System. pp 3-28. A. Iggo (ed). Springer, New York.

Angaut-Petit, D. (1975a). The dorsal column system: I. Existence of long ascending postsynaptic fibres in the cat's fasciculus gracilis. Exp. Brain Res. 22: 457-70.

Angaut-Petit, D. (1975b). The dorsal column system II. Functional properties and bulbar relay of the postsynaptic fibres of the cat's fasciculus gracilis. Exp. Brain Res. 22: 471-493.

Arai, H. and Emson, P.C. (1986). Regional distribution of Neuropeptide K and other tachykinins (neurokinin A, neurokinin B and substance P) in the rat central nervous system. Brain Res. 399: 240-249.

Arkinstall, S., Emergy, I., Church, D., Chollet, A. and Kawashima, E. (1994). Calcium influx and protein kinase Cα activation mediate arachadonic acid mobilisation by the human NK_2 receptor expressed in Chinese hamster ovary cells. FEBS Letts. 338: 75-80.

Bannon, M.J., Poosch, M.S., Haverstick, D.M., Mandal, A., Xue, I.C.H., Shibata, K. and Dragovic, L. (1992). Preprotachykinin gene expression in the human basal ganglia: Characterisation of mRNAs produced by alternate RNA splicing. Mol. Brain Res. 12: 225-231.

Basbaum, A.I., Gautron, M., Jazat, F., Mayes, M. and Guilbaud, G. (1991). The spectrum of fibre loss in a model of neuropathic pain in the rat: an electron microscope study. Pain 47(3): 359-367.

Beal, J.A. and Bicknell, H.R. (1981). Primary afferent distribution pattern in the marginal zone (Lamina I) of the adult monkey and cat lumbosacral spinal cord. J. Comp. Neurol. 202: 255-263.

Beal, J.A. and Fox, C.A. (1976). Afferent fibres in the substantia gelatinosa of the adult monkey (*Macaca Mulatta*) : A golgic study. J. Comp. Neurol. 168: 113-144.

Beck, P.W., Handwerker, H.O. and Zimmermann, M. (1974). Nervous outflow from the cat's foot during noxious radiant heat stimulation. Brain Res. 67: 373-386.

Bennett, G.J. and Xie, Y.K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33: 87-107.

Bennett, G.J., Abdelmoumene, M., Hayashi, H., Hoffert, M.J., Dubner, R. (1981). Spinal cord layer 1 neurons with axon collaterals that generate local arbors. Brain Res. 209: 421-26.

Beresford, I.J.M, Birch, P.J., Hagan, R.M. and Ireland, S.J. (1991). Investigation into species variants in tachykinin NK_1 receptors by use of the non-peptide antagonist CP-96,345. Br. J. Pharmacol. 104: 292-293.

Beresford, I.J.M., Ireland, S.J., Stables, J.M., Stubbs, C.M., Ball, D., Hagan, R.M. and Birch, P.J. (1992). Investigation into differences in tachykinin NK_1 receptors between and within species using a peptide and a non-peptide NK_1 receptor antagonist. Br. J. Pharmacol. 106: 92P.

Bergstrom, L., Torrens, Y., Saffroy, M., Beaujouan, J.C., Lavielle, S., Chassaing, G., Morgat, J.L., Glowinski, J. and Marquet, A. (1987). [^{3}H] Neurokinin B and [^{125}I]Bolton-Hunter eledoisin label identical tachykinin binding sites in the rat brain and peripheral tissue. J. Neurochem. 48: 125-133.

Besson, J-M. and Chaouch, A. (1987). Peripheral and spinal mechanisms of nociception. Physiol. Rev. 67: 67-186.

Besson, J.M., Guilbaud, G. and Le Bars D. (1975). Descending inhibitory influences exerted by the brainstem upon the activities of dorsal horn lamina V cells induced by intra-arterial injection of bradykinin into the limbs. J. Physiol. 248: 725-39.

Bessou, P. and Perl, E.R. (1969). Response of cutaneous sensory units with unmyelinated fibres to noxious stimuli. J. Neurophysiol. 32: 1025-1043.

Bettaney, J., Dray, A. and Perkins, M.N. (1988). Protein kinase C stimulates afferent Aδ and C fibres in neonatal rats in vitro. J. Physiol. 396: 160P.

Birch, P.J., Harrison, S.M., Hayes, A.G., Rogers, H. and Tyers, M.B. (1992). The non-peptide NK_1 receptor antagonist (±)CP-96,345 produces antinociceptive and anti-oedema effects in the rat. Br. J. Pharmacol. 105: 508-510.

Bliss, T.V.P. and Lomo, T. (1973). Long lasting potentiation of synaptic transmission in the dentate area of the anaesthetised rabbit following stimulation of the perforant path. J. Physiol. 232: 331-356.

Blix, M. (1884). Experimentelle Beitrage zur Losung der Frage uber die Specifishe Energie der Hautnerven. Z. Biol. 20: 141-156. Cited from Sinclair (1967).

Blumberg, P.M., Jaken, S., Konig, B., Sharkey, N.A., Leach, K.L., Jenq, A.Y. and Yeh, E. (1984). Mechanisms of action of the phorbol ester tumour promotors: specific receptors for lipophillic ligands. Biochem. Pharmacol. 33: 933-940.

Bodian, D. (1962). The generalised vertebrate neuron. Science 137: 323-326.

Bonner, T.I., Affolter, H-V., Young, A.C. and Young, W.S. (1987). A cDNA encoding the precursor of the rat neuropeptide neurokinin B. Mol. Brain Res. 2: 243-249.

Bristow, D.R., Curtis, N.R., Suman-Chauhan, N., Watling, K.J. and Williams, B.J. (1987). Effects of tachykinins on inositol phospholipid hydrolysis in slices of hamster urinary bladder. Br. J. Pharmacol. 90: 211-217.

Brodal, A. and Rexed, B. (1953). Spinal afferents to the lateral cervical nucleus in the cat. J. Comp. Neurol. 98: 199-211.

Brown, A.G. (1971). Effects of descending impulses on transmission through the spinocervical tract. J. Physiol. 219: 103-125.

Brown, A.G. (1981a). Organisation in the spinal cord : The Anatomy and Physiology of Identified neurons. Springer-Verlag. Berlin

Brown, A.G. (1981b). The Primary Afferent Input : Some basic principles. In : Spinal Cord Sensation. Eds. A.G. Brown and M. Retheyli. P 23-43.

Brown, A.G. and Fyffe, R.E.W. (1981). Form and function of dorsal horn neurons with axons ascending the dorsal columns in cat. J. Physiol. 321: 31-47.

Brown, A.G. and Iggo, A. (1967). A quantitative study of cutaneous receptors and afferent fibres in the cat and rabbit. J. Physiol. 193: 707-733.

Brown, A.G., House, C.R., Rose, P.K. and Snow, P.J. (1976). The morphology of spinocervical tract neurons in the cat. J. Physiol. 260: 719-738.

Brown, A.G., Rose, P.K. and Snow, P.J. (1977). The morphology of spinocervical tract neurons revealed by intracellular injection of horseradish peroxidase. J. Physiol. 270: 747-764.

Brown, A.G., Rose, P.K. and Snow, P.J. (1980a). Dendritic trees and cutaneous receptive fields of adjacent SCT neurons in the cat. J. Physiol. 300: 429-40.

Brown, A.G., Fyffe, R.E.W. and Nobel, R. (1980b). Projections from Pacinican corpuscles and rapidly adapting mechanoreceptors of glabrous skin to the cat's spinal cord. J. Physiol. 301: 385-400.

Brown, A.G., Brown, P.B., Fyffe, R.E.W., Pubols, L.M. (1983). Receptive field organisation and response properties of spinal neurons with axons ascending the dorsal columns in the cat. J. Physiol. 337: 575-588.

Buck, S.H., Helke, C.J., Burcher, E., Shults, L.W. and O'Donohue, T.L. (1986). Pharmacologic characterisation and autoradiographic distribution of binding sites for iodinated tachykinins in the rat central nervous system. Peptides 7: 1109-1120.

Buck, S.H., Harbesan, S.L., Hassmann, C.F., Shatzer, S.A., Rouissi, N., Nantel, F. and Giersbergen, P.L.M. (1990). [$Leu^{9}(CH_2NH)Leu^{10}$]-neurokinin A (4-10) (MDL 28,564) distinguishes tissue tachykinin NK_2 receptors. Life Sci. 47: PL37-PL41.

Bullit, E. (1989). Induction of c-fos like protein within the lumbar spinal cord and thalamus of the rat following peripheral stimulation. Br. Res. 391-397.

Bullit, E. (1991). Somatotopy of spinal nociceptive processing. J. Comp. Neurol. 312: 279-290.

Burgess, P.R. and Perl, E.R. (1967). Myelinated afferent fibres responding specifically to noxious stimulation of the skin. J. Physiol. 190: 541-562.

Burgess, P.R. and Perl, E.R. (1973). Cutaneous mechanoreceptors and nociceptors. Handbook of Sensory Physiology II: Somatosensory System. Ed. A. Iggo. Springer-Verlag, Berlin. P29-78.

Burgess, P.R., Petit, D. and Warren, R.M. (1968). Receptor types in cat hairy skin supplied by myelinated fibres. J. Neurophysiol. 31: 833-848.

Burgess, G.M., Mullaney, I., McNeill, M., Dunn, P.M. and Rang, H.P. (1989). Second messengers involved in the mechanism of action of bradykinin in sensory neurons in culture. J. Neuroscience 9(9): 3314-3325.

Calcagnetti, D.J., Fleetwood, S.W. and Holtzman, S.G. (1990). Pharmacological profile of the potentiation of opioid analgesia by restraint stress. Pharmacology, Biochemistry and Behaviour 37(1): 193-9.

Cameron, A.A., Leah, J.D. and Snow, P.J. (1988). The co-existence of neuropeptides in feline sensory neurons. Neuroscience 27: 969-999.

Campbell, J.N. and Meyer, R.A. (1983). Sensitisation of unmyelinated nociceptive afferents in monkey varies with skin type. J. Neurophysiol. 49: 98-110.

Campbell, J.N., Mayer, R.A. and LaMotte, R.H. (1979). Sensitisation of myelinated nociceptive afferents that innervate monkey hand. J. Neurophysiol. 42(6): 1669-1679.

Carr, P.A. and Nagy, J.I. (1993). Emerging relationships between cytochemical properties and sensory modality transmission in primary sensory neurons. Brain. Res. Bulletin 30: 209-219.

Carter, M.S. and Krause, J.E. (1990). Structure expression and some regulatory mechanisms of the rat preprotachykinin gene encoding substance P, neurokinin A, neuropeptide K and neuropeptide γ. J. Neuroscience. 10: 2203-2214.

Castagna, M., Takai, Y., Kaibuchi, K., Sario, K., Kikawa, U. and Nishizuka, Y. (1982). Direct activation of calcium activated, phospholipid dependant protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257: 7847-7851.

Cerne, R., Rusin, K.I. and Randic, M. (1993). Enhancement of the N-methyl-D-aspartate response in spinal dorsal horn neurons by c-AMP-dependent protein kinase. Neuroscience Letts. 161: 124-128.

Cervero, F. and Iggo, A. (1980). The substantia gelatinosa of the spinal cord. A critical review. Brain 103: 717-772.

Cervero, F., Iggo, A. and Ogawa, H. (1976). Nociceptor-driven dorsal horn neurons in the lumbar spinal cord of the cat. Pain 2: 5-24.

Cervero, F., Iggo, A. and Molony, V. (1977). The morphology of spinocervical tract neurons revealed by intracellular injection of horseradish peroxidase (cat). J. Physiol. 270: 747-64.

Cervero, F., Handwerker, H.O. and Laird, J.M.A. (1988). Prolonged noxious stimulation of the rat's tail: responses and encoding properties of dorsal horn neurons. J. Physiol. 404: 419-436.

Chambers, M.R., Andres, K.H., During, M. Von and Iggo, A. (1972). The structure and function of the slowly adapting type II mechanoreceptors in hairy skin. Quart. J. Exp. Physiol. 57: 417-445.

Chang, M.M., Leeman, S.E. and Niall, H.D. (1971). Amino acid sequence of substance P. Nature (New Biol.) 232: 86-87.

Chapman, V. and Dickenson, A.H. (1993). The effect of intrathecal administration of RP 68580, a potent neurokinin-1 antagonist on nociceptive transmission in the rat spinal cord. Neuroscience Letts 157: 149-152.

Chaouch, A., Menetrey, D., Binder, D. and Besson, J.M. (1983). Neurons at the origin of the medial component of the bulbopontine spinoreticular tract in the rat: An anatomical study using horseradish peroxidase retrograde transport. J. Comp. Neurol. 214: 309-320.

Charney, Y., Paulin, C., Chayvialle, J.A. and Dubois, P.M. (1983). Distribution of substance P-like immunoreactivity in the spinal cord and dorsal root ganglia of human foetus and infant. Neuroscience 10: 41-55.

Chen, L. and Huang, L-I.M. (1992). Protein kinase C reduces Mg^{2+} block of NMDA receptor channels as a mechanism of modulation. Nature 356: 521-523.

Chi, S-I., Levine, J.D. and Basbaum, A.I. (1993). Peripheral and central contributions to the persistent expression of spinal cord fos-like immunoreactivity produced by sciatic nerve transection in the rat. Brain Res. 617: 225-237.

Cleland, C.L., Lim, F.-Y. and Gebhart, G.F. (1994). Pentobarbitol prevents the development of C-fibre-induced hyperalgesia in the rat. Pain 57: 31-43.

Coderre, T.J. (1992). Contribution of protein kinase C to central sensitisation and persistent pain following tissue injury. Neuroscience Letts. 140: 181-184.

Coderre, T.J. and Melzack, R. (1987). Cutaneous hyperalgesia: contributions of the peripheral and central nervous systems to the increase in pain sensitivity after injury. Brain Res. 404: 95-106.

Coderre, T.J. and Melzack, R. (1991). Central neural mediators of secondary hyperalgesia following heat injury in rats: neuropeptides and excitatory amino acids. Neuroscience Letts. 131: 71-74.

Coderre, T.J. and Melzack, R. (1992). The role of NMDA receptor-operated calcium channels in persistent nociception after formalin-induced tissue injury. J. Neurosci. 12: 3671-3675.

Coderre, T.J. and Yashpal, K. (1994). Intracellular messengers contributing to persistent nociception and hyperalgesia induced by L-glutamate and substance P in the rat formalin pain model. Eur. J. Neurosci. 6: 1328-1334.

Coderre, T.J., Vaccarino, A.L. and Melzack, R. (1990). Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res. 535: 155-158.

Coderre, T.J., Pitcher, G.M. and Yashpal, K. (1993). Pharmacological and anatomical evidence for a contribution of protein kinase C to persistent pain and hyperalgesia following tissue injury in rats. 7th World Congress on Pain Abstracts. IASP Publications. Seattle, P223.

Coderre, T.J., Yashpal, K., Pitcher, G.M. and Quirion, R. (1994). Anatomical and pharmacological evidence for a contribution of protein kinase C to persistent nociception and hyperalgesia after noxious chemical and thermal stimulation. Proceedings of the 7th World Congress on Pain. Prog. in Pain Res. and Management Vol 2. G.F. Gebhart, D.L. Hammond and T.S. Jensen (Eds). IASP Press, Seattle.

Corbett, A., Paterson, S., McKnight, A., Magnan, J. and Kosterlitz, H. (1982). Dynorphin 1-8 and dynorphin 1-9 are ligands for the κ-subtype of opiate receptors. Nature 299: 79-81.

Craig, A.D. (1978). Spinal and medullary input to the lateral cervical nucleus. J. Comp. Neurol. 181: 729-44.

Craig, A.D. and Kniffki, K.D. (1985). Spinothalamic lumbosacral lamina I cells responsive to skin and muscle stimulation in the cat. J. Physiol. 365: 197-221.

Craig, A.D., Linnington, A.J. and Kniffki, K.D. (1989a). Significant differences in the retrograde labelling of spinothalamic tract cells by horseradish peroxidase and the fluorescent tracers, fast blue and diamidino yellow. Exp. Brain Res. 74: 431-436.

Craig, A.D., Linnington, A.J. and Kniffki, K.D. (1989b). Cells of origin of spinothalamic tract projections to the medial and lateral thalamus in the cat. J. Comp. Neurol. 289: 568-585.

Cridland R.A. and Henry, J.L. (1986). Comparison of the effects of substance P, neurokinin A, physalaemin and eledoisin in facilitating a nociceptive reflex in the rat. Brain Res. 381: 93-99.

Cridland, R.A. and Henry, J.L. (1988). Facilitation of the tail-flick by noxious cutaneous stimulation in the rat: antagonism by a substance P analogue. Brain Res. 462: 15-21.

Croze, S., Ducleux, R. and Kenshalo, D.R. (1976). The thermal sensitivity of the polymodal nociceptors in the monkey. J. Physiol. 263: 539-562.

Cuello, A.C., Jessell, T.M., Kanazawa, I. and Iverson, L.L. (1977). Substance P: Localisation in synaptic vesicles in rat central nervous system. J. Neurochem. 29: 747-751.

Curran, T., Gordon, M.B., Rubino, K.L. and Sambucetti, L.C. (1987). Isolation and characterisation of the c-fos (rat) cDNA and analysis of post-translational modification *in vitro*. Oncogene 2: 79-84.

Dado, R.J., Katter, J.T. and Giesler, G.J. (1994a). Spinothalamic and spinohypothalamic tract neurons in the cervical enlargement of rats I. Locations of antidromically identified axons in the thalamus and hypothalamus. J. Neurophysiol. 71(3): 959-80.

Dado, R.J., Katter, J.T. and Giesler, G.J. (1994b). Spinothalamic and spinohypothalamic tract neurons in the cervical enlargement of rats II. Responses to innocuous and noxious mechanical and thermal stimuli. J. Neurophysiol. 71(3): 981-1002.

Dado, R.J., Katter, J.T. and Giesler, G.J. (1994c). Spinothalamic and spinohypothalmic tract neurons in the cervical enlargement of rats III. Locations of antidromically identified axons in cervical cord white matter. J. Neurophysiol. 71(3): 1003-21.

Dalsgaard, C-J., Haegerstrand, A., Theodorsson-Norheim, E., Brodin, E. and Hokfelt, T. (1985). Neurokinin A-like immunoreactivity in rat primary sensory neurons: coexistence with substance P. Histochemistry 83: 37-39.

Dam, T-V., Takeda, Y., Krause, J.E., Escher, E. and Quirion, R. (1990). Preprotachykinin (72-92) peptide amide: an endogenous preprotachykinin 1 gene-derived peptide that preferentially binds to neurokinin 2 receptors. Proc. Natl. Acad. Sci. (USA) 87: 246-250.

D'Amour, F.E. and Smith, D.L. (1941). A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther. 72: 74.

Davies, J. and Dray, A. (1980). Depression and facilitation of synaptic responses in cat dorsal horn by substance P administered into the substantia gelatinosa. Life Sci. 27: 2037-2042.

DeBiasi, S. and Rustioni, A. (1988). Glutamate and substance P co-exist in primary afferent terminals in the superficial laminae of the spinal cord. Proc. Natl. Acad. Sci. (USA) 85: 7820-7824.

DeKoninck, Y. and Henry, J.L. (1991). Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons *in vivo* by noxious stimulation. Proc. Natl. Acad. Sci. (USA). 88: 11344-1348.

DeRiemer, S.A., Strong, J.A., Albert, K.A., Greenguard, P. and Kaczmarek, L.K. (1985). Enhancement of calcium current in Aplysia by phorbol ester and protein kinase C. Nature 313-316.

Dickenson, A.H. (1990). A cure for wind-up: NMDA receptor antagonists as potential analgesics. Trends Pharmacol. Sci. 11: 307-309.

Doi, T. and Jurna, I. (1981). Intrathecal substance P depresses the tail-flick response-antagonism by naloxone. Naunyn-Schmied. Arch. Pharmacol. 317: 135.

Donaldson, L.F., Harmar, A.H., McQueen, D.S. and Seckl. J.R. (1992). Increased expression of preprotachykinin, calcitonin gene-related peptide but not vasoactive intestinal peptide messenger RNA in dorsal root ganglia during the development of adjuvant monoarthritis in the rat. Mol. Brain Res. 16: 143-149.

Doucette, R., Theriault, E. and Diamond, J. (1987). Regionally selective elimination of cutaneous thermal nociception in rats by neonatal capsaicin, J. Comp. Neurol. 261: 583-591.156

Dougherty, P.M. and Willis, W.D. (1992). Enhancement of spinothalamic neuron responses to chemical and mechanical stimuli following combined

microiontophoretic application of N-methyl-D-aspartic acid and substance P. Pain 47: 85-93.

Dougherty, P.M. and Willis W.D. (1992). Enhanced responses of spinothalamic tract neurons to excitatory amino acids accompanying capsaicin-induced sensitisation in the monkey. J. Neuroscience 12(3): 833-894.

Dougherty, P.M., Palecek, J., Paleckova, V. and Willis, W.D. (1994). Neurokinin 1 and neurokinin 2 antagonists attenuate the responses and NK_1 antagonists prevent the sensitisation of primate spinothalamic tract neurons after intradermal capsaicin. J. Neurophysiol. 72: 1464-1475.

Douglas, W.W., Ritchie, J.M. and Straub, R.W. (1960). The role of non-myelinated fibres in signalling cooling of the skin. J. Physiol. 150: 266-283.

Downie, J.W., Ferrington, D.G., Sorkin, L.S. and Willis, W.D. (1988). The primate spinocervicothalamic pathway: Responses of cells of the lateral cervical nucleus and spinocervical tract to innocuous and noxious stimuli. J. Neurophysiol. 59: 861-885.

Draisci, G. and Iadarola, M.J. (1989). Temporal analysis of increases in c-fos, preprodynorphin and preproenkephalin mRNAs in rat spinal cord. Mol. Brain Res. 6: 31-37.

Dray, A., Bettaney, J., Forster, P. and Perkins, M.N. (1988). Bradykinin-induced stimulation of afferent fibres is mediated through protein kinase C. Neuroscience Letts. 91: 301-307.

Dubner, G. (1991). Neuronal plasticity and pain following peripheral tissue inflammation or nerve injury. In M.R. Bond, J.E. Charlton and C.J. Woolf (Eds). Pain Research and Clinical Management, Vol. 4, Proc. VIth World Congress on Pain, Elsevier, Amsterdam, 1991, 263-276.

Dubner, R. and Bennett, G.J. (1983). Spinal and trigeminal mechanisms of nociception. Ann. Rev. Neurosci. 6: 381-418.

Dubner, R. and Ruda, M.A. (1992). Activity dependent neuronal plasticity following tissue injury and inflammation. T.I.N.S. 15: 96-103.

Dubner, R., Kenshallo, D.R., Maixner, W., Bushnell, C. and Oliveras, J-L. (1989). The correlation of monkey medullary dorsal horn neuronal activity and the perceived intensity of noxious heat stimuli. J. Neurophysiol. 62(2): 450-457.

Duclaux, R. and Kenshallo, D.R. (1972). The temperature sensitivity of type I slowly adapting mechanoreceptors in cats and monkeys. J. Physiol. 224: 647-664.

Duggan, A.W., Hall, J.G. and Headley, P.M. (1977). Enkephalins and dorsal horn neurons of the cat: Effects on responses to noxious and innocuous skin stimuli. Br. J. Pharmacol. 61 : 399-408.

Duggan, A.W., Griersmith, B.T., Headley, P.M. and Hall, J.G. (1979). Lack of effect by substance P at sites in the substantia gelatinosa where met-enkephalin reduces the transmission of nociceptive impulses. Neuroscience Letts. 12: 313-317.

Duggan, A.W., Griersmith, B.T. and Johnson, S.M. (1981). Supraspinal inhibition of the excitation of dorsal horn neurons by strychnine and bicuculine. Brain Res. 210: 231-241.

Duggan, A.W., Morton, C.R., Zhao, Z.Q. and Henry, I.A. (1987). Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the rat; a study with antibody microprobe. Brain Res. 403: 345-349

Duggan, A.W., Henry, I.A., Morton, C.R., Hutchison, W.D. and Zhao, Z.Q. (1988). Cutaneous stimuli release immunoreactive substance P in the dorsal horn of the cat. Brain Res. 451: 261-273.

Duggan, A.W., Hope, P.J., Jarrott, B., Schaible, H-G. and Fleetwood-Walker, S.M. (1990). Release, spread and persistence of neurokinin A in the dorsal horn of the cat following noxious cutaneous stimulation. Studies with antibody microprobes. Neuroscience 35(1): 195-202.

Duggan, A.W., Schaible, H-G., Hope, P.J. and Lang, C.W. (1992). Effect of peptidase inhibition on the pattern of intraspinally released immunoreactive substance P detected with antibody microprobes. Brain Res. 579: 261-269.

Edinger, L. (1889). Verleichend-entwicklungsgeschichtliche und anatomisch studien im Bereiche des Centralnervousystems 2. Uber die Fortsetzung der hinteren Ruckenmarkswurzdn zum Gehirn. Anat. Anz., 4: 121-128. Cited from Willis *et al,* (1979).

Elde, R., Schalding, M., Ceccatelli, S., Nakanishi, S. and Hokfelt, T. (1990). Localisation of neuropeptide receptor mRNA in rat brain: initial observation using probes for neurotensin and substance P receptors. Neurosci. Letts. 120: 134-138.

Elliott, P.J., Smith, G.D., Bowers, J.S., Wiseman, J., Cunoosamy, M.P., Harrison, S.M., Beresford, I.J.M. and Birch, P.J. (1992). Antinociceptive effects of the non-peptide NK_1 antagonist (±)CP-99,994 in the gerbil. Neuropeptides 24(4): C15.

Emonds-Alt, X., Vilain, P., Goulaouic, P., Proietto, D., Van Broeck, D., Advenier, C., Naline, E., Neliat, G., Le Fur, G. and Breliere, J.C. (1992). A potent and

selective non-peptide antagonist of the neurokinin A (NK_2) receptor. Life Sci. 50: 101-106.

Emonds-Alt, X., Golliiot, F., Pointeau, P., Le Fur, G., Breliere, J.C. (1993). Characterisation of the binding sites of [^{3}H]SR 48968, a potent non-peptide radioligand antagonist of the neurokinin-2 receptor. Biochem. Biophys. Res. Commun. 191: 1172-1177.

Enevoldson, T.P. and Gordon, G. (1984). Postsynaptic dorsal column neurons in the cat: A study with retrograde transport of horseradish peroxidase. Exp. Brain Res. 75: 611-20.

Erdos, E.G. (1979). Bradykinin, kallidin and kallikrein. In Handbook of Experimental Pharmacology. Vol 25. Springer-Verlag, Berlin.

Erspamer, V. (1981). The tachykinin peptide family. T.I.N.S. 4: 267-269.

Fardin, V. and Garret, C. (1991). Species differences between [^{3}H]substance P binding in rat and guinea-pig shown by use of peptide agonists and antagonists. Eur. J. Pharmacol. 201: 231-234.

Fardin, V., Bock, M.D., Foucault, F., and Garret, C. (1993). RP 67580, a non-peptide substance P antagonist with low penetration to the central nervous system. IASP 7th World Congress on Pain Abstract: 636 P229.

Ferrell, W.R., Wood, L. and Baxendale, R.H. (1988). The effect of acute joint inflammation on flexion excitability in the decerebrate, low spinal cord. Q. J. Exp. Physiol. 73: 95-102.

Fields, H.L., Wagner, G.W. and Anderson S.D. (1975). Some properties of spinal neurons projecting to the medial brainstem reticular formation. Exp. Neurol. 47: 118-134.

Fields, H.L., Clanton, C.H. and Anderson, S.D. (1977). Somatosensory properties of spinoreticular neurons in the cat. Br. Res. 120: 49-66.

Fitzgerald, M. (1983). Capsaicin and sensory neurons- a review. Pain 15: 109-130.

Fitzgerald, M. (1984). The course and termination of primary afferent fibres. The Textbook of Pain: P.D. Wall and R. Melzack (eds). P34-48.

Fitzgerald, M. and Lynn, B. (1977). The sensitisation of high threshold mechanoreceptors with myelinated axons by repeated heating. J. Physiol. 265: 549-563.

Fleetwood-Walker, S.M., Mitchell, R., Hope, P.J., El-Yassir, N. and Molony, V. (1987). Neurokinin A as a transmitter of primary afferents involved in thermal nociception. In J.L. Henry, R. Couture, A.C. Cuello, G. Pelletier, R. Quirion and D. Regoli (Eds). Substance P and Neurokinins. Springer, New York P239-240.

Fleetwood-Walker, S.M., Mitchell, R., Hope, P.J., El-Yassir, N., Molony, V. and Bladon, C.M. (1990). The involvement of neurokinin receptor subtypes in somatosensory processing in the superficial dorsal horn of the cat. Brain Res. 519: 169-182.

Fleetwood-Walker, S.M., Hope, P.J., Parker, R.M.C. and Mitchell, R. (1991). Effects of antagonists selective for neurokinin-1 and neurokinin-2 receptors on somatosensory responses of rat dorsal horn neurons. Br. J. Pharmacol. 104: 472.

Fleetwood-Walker, S.M., Mitchell, R., Hope, P.J., El-Yassir, N., Molony, V. and Bladon, C.M. (1992). The involvement of neurokinin receptor subtypes in somatosensory processing in the superficial dorsal horn of the cat: corrigendum. Brain Res. 579: 357.

Fleetwood-Walker, S.M., Parker, R.M.C., Munro, F.E., Hope, P.J. and Mitchell, R. (1993). The role of tachykinins and somatostatin in spinal nociceptive processing. The Journal of Pain Society 11: 22-26.

Fong, T.M., Anderson, S., Yu, H., Huang, R.P.C. and Strader C.D. (1992). Differential activation of intracellular effector by two isoforms of human neurokinin 1 receptor. Mol. Pharmacol. 41: 24-30.

Foreman, R.D., Applebaum, A.E., Beall, J.E., Trevion, D.L. and Willis W.D. (1975). Response of primate spinothalamic tract neurons to electrical stimulation of hindlimb peripheral nerves. J. Neurophysiol. 38: 132-145.

Franck, J., Brodin, E., Fried, K., Rosen, A., Yamamoto, Y. and Fried, G. (1991). The effect of selective serotonin neurotoxin treatment on tachykinin levels in the rat ventral spinal cord. Neuroscience 45(2): 339-345.

Freedman, J., Post, C., Kahrstrom, J., Ohlen, A., Mollenholt, P., Owman, C., Alari, L. and Hokfelt, T. (1988). Vasoconstrictor effects in spinal cord of the substance P antagonist [D-Arg^{1}, D-Trp^{7,9},Leu^{11}]-substance P and somatostatin and interaction with thyrotropin releasing hormone. Neuroscience 27: 267-278.

Frenk, H., Bossut, D., Urca, G. and Mayer, D.J. (1988). Is substance P a primary afferent transmitter for nociceptive input? I. Analysis of pain-related behaviours resulting from intrathecal administration of substance P and six excitatory compounds. Brain Res. 455: 223-231.

Fyffe, R.E.W. (1984). Afferent Fibres. In : Handbook of the Spinal Cord. Volumes 2 and 3 : Anatomy and Physiology. R.A. Davdoff (Ed). Mercel Dekker Inc. New York and Basel.

Gamse, R. and Saria, A. (1986). Nociceptive behaviour after intrathecal injections of substance P, neurokinin A and calcitonin gene-related petide in mice. Neuroscience Letts. 70: 143-147.

Garces, Y.I., Rabito, S.F., Minshall, D. and Sagen, J. (1992). Lack of potent antinociceptive activity by substance P antagonist CP-96,345 in the rat spinal cord. Life Sciences 52: 353-360.

Garret, C., Carruette, A., Fardin, V., Moussaoui, S., Pey-Ronnel, J-F., Blanchard, J-C. and Laduron, P.M. (1991). Pharmacological properties of a potent and selective non-peptide substance P antagonist. Proc. Natl. Acad. Sci. USA. 88: 10208-10212.

Garry, M.G. and Hargreaves, K.M. (1992). Enhanced release of immunoreactive CGRP and substance P from spinal dorsal horn slices occurs during carrageenan inflammation. Br. Res. 582: 139-142.

Georgopoulis, A.P. (1976). Functional properties of primary afferent units probably related to pain mechanisms in primate glabrous skin. J. Neurophysiol. 39: 71-83.

Gerber, G., Kangrga, I., Ryu, P.D., Larew, J.S.A. and Randic, M. (1989). Multiple effects of phorbol esters in the rat spinal dorsal horn. J. Neuroscience 9(10): 3606-3617.

Gibson, S.J., Polak, J.M., Bloom, S.R. and Wall, P.D. (1981). The distribution of nine peptides in rat spinal cord with special emphasis on substantia gelatinosa and on the area around the central canal. J. Comp. Neurol. 201: 65-79.

Giesler, G.J. and Cliffer, K.D. (1985). Postsynaptic dorsal column pathway of the rat II. Evidence against an important role in nociception. Br. Res. 326: 347-56.

Giesler, G.J., Urca, G., Cannal, J.T. and Liebskind, J.C. (1979). Response properties of neurons of the lateral cervical nucleus in the rat. J. Comp. Neurol. 98: 199-211.

Giesler, G.J., Nahin, R.L. and Madsen, A.M. (1984). Postsynaptic dorsal column pathway of the rat I. Anatomical studies. J. Neurophysiology 51(2): 260-75.

Gitter, B.D., Waters, D.C., Bruns, R.F., Mason, W.R., Nixon, J.A. and Howbert, J.J. (1991). Species differences in affinities of non-peptide antagonists for substance P receptors. Eur. J. Pharmacol. 197: 237-238.

Go, V.L.W. and Yaksh, T.L. (1987). Release of substance P from the cat spinal cord. J. Physiol. 391: 141-167.

Gobel, S. (1978). Golgic studies of the neurons in layer II of the dorsal horn of the medulla (trigeminal nucleus caudalis). J. Comp. Neurol. 180: 395-414.

Gobel, S. and Binck, J.M. (1977). Degenerative changes in primary trigeminal axons and in neurons in nucleus caudalis following tooth pulp extirpations in the cat. Br. Res. 132: 347-354.

Gobel, S., Falls, W.M., Bennett., G.J., Hayashi, H. and Humphrey, E. (1980). An EM analysis of the synaptic connections of horseradish peroxidase - filled stalk cells and islet cells in the substantia gelatinosa of adult cat spinal cord. J. Comp Neurol. 194: 781-807.

Goldscheider, A. (1884). The specific energy of the sensory nerves of the skin. In: Classical German Contributions to Pain Research. H.O. Handwerker and K. Brune (Eds). (1987). Tagblatt-Druckerei KG. pp 49-67.

Goodchild, A.K., Dampney, R.A.L., Bandler, R. (1982). A method of evoking physiological responses by stimulation of cell bodies, but not axons of passage, within localised regions of the central nervous system. J. Neurosci. Methods 6: 351-363.

Guard, S. and Watson, S.P. (1991). Tachykinin receptor types: classification and membrane signalling mechanisms. Neurochem. Int. 18(2): 149-165.

Hagan, R.M., Ireland, S.J., Bailey, F., McBride, C., Jordan, C.C. and Ward, P. (1991). A spirolactam conformationally-constrained analogue of physalaemin which is a peptidase-resistant, selective neurokinin NK_1 receptor antagonist. Br. J. Pharmacol. (1991) 102: 168P.

Hammond, D.L. and Ruda, M.A. (1991). Developmental alterations in the distribution of immunoreactive calcitonin-gene-related peptide, substance P and fluoride resistant phosphatase in the spinal cord of the rat after neonatal administration of capsaicin: relationship to nociceptive threshold. J. Comp. Neurol. 312: 436-350.

Handwerker, H.O., Iggo, A. and Zimmermann, M. (1975). Segmental and supraspinal actions on dorsal horn neurons responding to noxious and non-noxious skin stimuli. Pain 1: 147-165.

Handwerker, H.O., Anton, F. and Reeh, P.W. (1987). Discharge pattern of afferent cutaneous nerve fibres from rats tail during prolonged noxious mechanical stimulation. Exp. Brain Res. 65: 493-504.

Hanley, M.R., Lee, C.M., Jones, L.M. and Michell, R.H. (1980). Similar effects of substance P and related peptides on salivation and on phosphatidylinositol turnover in rat salivary glands. Mol. Pharmacol. 18: 78-83.

Harmar, A.J. and Keen, P. (1984). Rat sensory ganglia incorporate radiolabelled amino acids into substance K (neurokinin α) in vitro. Neuroscience Letts. 51: 387-391.

Harmar, A.J., Hyde, V. and Chapman, K. (1990). Identification and cDNA sequence of δ-preprotachykinin, a fourth splicing variant of the rat substance P precursor. FEBS Lett. 275: 22-24.

Harris, N.C. and Ryall, R.W. (1988). Mustard oil excites but does not inhibit nociceptive dorsal horn neurons in the rat: a presumed effect on A-delta fibres. Br. J. Pharmacol. 94: 180-184.

Hayes, R.L., Bennett, G.J., Newlan, P. and Mayer, D.J. (1976). Analgesic effects of certain noxious and stressful manipulations in the rat. Soc. Neurosci. Abstr. 2: 1350.

Hayes, A.G., Sheenan, M.J. and Tyers, M.B. (1987). Differential sensitivity of models of antinociception in the rat, mouse and guinea-pig to μ and κ-opioid receptor agonists. Br. J. Pharmacol. 91: 823-832.

Hayes, R.L., Mao, J., Price, D.D., Germano, A., D'Avella, D., Fiori, M. and Mayer, D.J. (1992). Pre-treatment with gangliosides reduces abnormal nociceptive responses associated with a rodent peripheral mononeuropathy. Pain 48: 391-396.

Heapy, C.G., Jamieson, A. and Russell, N.J.W. (1987). Afferent C-fibre and A-delta fibre activity in models of inflammation. Br. J. Pharmacol 90: 164P.

Helke, C.J., Charlton, C.G. and Wiley, R.G. (1986). Studies on the cellular localisation of spinal cord substance P receptors. Neuroscience 19: 523-533.

Helke, C.J., Krause, J.E., Mantyh, P.W., Couture, R. and Bannon, M.J. (1990). Diversity in mammalian tachykinin peptidergic neurons: multiple peptides, receptors and regulatory mechanisms. FASEB J. 4: 1606-1615.

Henry, J.L. (1976). Effects of substance P on functionally identified units in cat spinal cord. Brain Res. 114: 439-451.

Henry, J.L. and Salter, M.W. (1987). Neurokinin A excites nociceptive and non-nociceptive dorsal horn neurons in the cat spinal cord. In J.L. Henry, R. Couture, A.C. Cuello, G. Pelletier, R. Quirion and D. Regoli (Eds). Substance P and Neurokinins. Springer, New York P239-240.

Hensel, H. (1966). Classes of receptor units predominantly related to thermal stimuli. Touch, Heat and Pain : A.V.S. de Reuck and J Knight (Eds). 275-290.

Hensel, H. (1973). Cutaneous Thermoreceptors. Handbook of Sensory Physiology II: Somatosensory System. Ed. A. Iggo. Springer-Verlag, Berlin. P79-110.

Hensel, H. and Zotterman, Y.(1951). The response of mechanoreceptors to thermal stimulation. J. Physiol. 115: 16-24.

Hensel, H., Iggo, A. and Witt, I. (1960). A qualitative study of sensitive cutaneous thermoreceptors with C afferent fibres. J. Physiol. 153: 113-126.

Herbert, J.N., Augereau, J.M., Gleye, J. and Maffrand, J.P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem. Biophys. Res. Commun. 172: 993-999.

Hershey, A.D. and Krause, J.E. (1990). Molecular characterisation of a functional cDNA encoding the rat substance P receptor. Science 247: 958-962.

Hill, R.G., Salt, T.E. and Leighton, G.E. (1985). Actions of putative substance P antagonists on neurons in the CNS. In R. Hakanson and F. Sundler (Eds). Tachykinin Antagonists. Elsevier, Amsterdam P377-382.

Hirakawa, M. and Kawata, M. (1992). Changes of chemoarchitectural organisation of the rat spinal cord following ventral and dorsal root transection. J. Comp. Neurol. 320: 339-352.

Hoffman, D.S., Dubner, R., Hayes, R.L. and Medlin, T.P. (1981). Neuronal activity in medullary dorsal horn of awake monkeys in a thermal discrimination task I. Responses to innocuous and noxious stimuli. J. Neurophysiol. 46(3): 409-27.

Hokfelt, T., Kellerth, J-O., Nilsson, G. and Pernow, B. (1975a). Experimental immunohistochemical studies on the localisation and distribution of substance P in cat primary sensory neurons. Brain Res. 100: 235-252.

Hokfelt, T., Kellerth, J., Nilsson, G. and Pernow, B. (1975b). Substance P: localisation in central nervous system and in some primary sensory neurons. Science 190: 889-890.

Hokfelt, T., Elde, R., Johansson, O., Luft, R., Nilsson, G. and Arimura, A. (1976). Immunohistochemical evidence for separate populations of somatostatin-containing and substance P-containing primary afferent neurons in the rat. Neuroscience 1: 131-134.

Hokfelt, T., Ljungdahl, A., Terenius, L., Elde, R. and Nilsson, G. (1977). Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: enkephalin and substance P. Proc. Natl. Acad. Sci. (USA) 74: 3081-3085.

Hong, S.K., Kniffki, K.D., Mense, S., Schmidt, R.F. and Wendison, M. (1979). Descending influences on the responses of spinocervical tract neurons to chemical stimulation of fine muscle afferents. J. Neurophysiol. 290: 129-140.

Hope, P.J., Jarrott, B., Schaible, H-G., Clarke, R.W. and Duggan, A.W. (1990). Release and spread of immunoreactive neurokinin A in the cat spinal cord in a model of acute arthritis. Brain Res. 533: 292-299.

Hua, X-Y, Theodorsson-Norheim, E., Brodin, E., Lundberg, J.M. and Hokfelt, T. (1985). Multiple tachykinins (neurokinin A, neuropeptide K and substance P) in capsaicin sensitive neurons in the guinea pig. Regul. Peptides 13: 1-19.

Huang, Y-Y., Colley, P.A. and Routtenberg, A. (1992). Postsynaptic then pre-synaptic protein kinase C activity may be necessary for long-term potentiation. Neuroscience 49: 819-827.

Hunt, S.P., Kelly, J.S., Emson, P.C., Kimmel, J.R., Miller, R.J. and Wu, J.Y. (1981). An immunohistochemical study of neuronal populations containing neuropeptides or δ-aminobutyrate within the superficial layers of the rat dorsal horn. Neuroscience 6: 1883-1898.

Hunt, S.P., Pini, A. and Evan, G. (1987). Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature 328: 632-634.

Hylden, J.L.K. and Wilcox, G.L. (1980). Intrathecal morphine in mice: a new technique. Eur. J. Pharmacol. 67: 313-316.

Hylden, J.L.K. and Wilcox, G.L. (1981). Intrathecal substance P elicits a caudally-directed biting and scratching behaviour in mice. Brain. Res. 217: 212-215.

Hylden, J.L., Hayashi, H., Bennett, G.J. and Dubner, R. (1985). Spinal lamina I neurons projecting to the parabrachial area of the cat midbrain. Brain Res. 336: 195-198.

Hylden, J.L., Hayashi, H., Dubner, R. and Bennett, G.J. (1986). Physiology and morphology of the lamina I spinomesencephalic projection. J. Comp. Neurol. 247: 505-515.

Hylden, J.L., Nahin, R.L., Traub, R.J. and Dubner, R. (1989). Expansion of receptive fields of spinal laminae I projection neurons in rats with unilateral

adjuvant-induced inflammation: the contribution of dorsal horn mechanisms. Pain 37: 229-243.

Hylden, J.L., Nahin, R.L., Traub, R.J. and Dubner, R. (1991). Effects of spinal kappa-opioid receptor agonists on the responsiveness of nociceptive superficial dorsal horn neurons. Pain 44: 187-193.

Hylden, J.L.K., Noguchi, K. and Ruda, M.A. (1992). Neonatal capsaicin treatment attenuates spinal fos activation and dynorphin gene expression following peripheral tissue inflammation and hyperalgesia. J. Neurosci. 12(5): 1716-1725.

Iadarola, M.J., Douglass, J., Civelli, O. and Naranjo, J.R. (1988). Differential activation of spinal cord dynorphin and enkephalin neurons during hyperalgesia: evidence using cDNA hybridisation. Brain Res. 455: 205-212.

Iggo, A. (1959). Cutaneous heat and cold receptors with slowly conducting (C) afferent fibres. Quart. J. Exp. Physiol. 44: 362-370.

Iggo, A. (1960). Cutaneous mechanoreceptors with afferent C fibres. J. Physiol. 152: 337-353.

Iggo, A. (1966). Cutaneous receptors with a high sensitivity to mechanical displacement. Touch, Heat and Pain : Eds A.V.S. de Reuck and J.Knight. P237-260.

Iggo, A. (1969). Cutaneous thermoreceptors in primates and sub-primates. J. Physiol 200: 403-430.

Iggo, A. (1974). Activation of cutaneous nociceptors and their actions on dorsal horn neurons. In : Advances in Neurology Vol. 4. J.J. Bonica (Ed). Raven Press, New York. pp 1-9.

Iggo, A. and Ogawa, H. (1977). Correlative physiological and morphological studies of rapidly adapting mechanoreceptors in cat's glabrous skin. J. Physiol. 266: 275-296.

Igwe, O.J. and Ning, L. (1994). Regulation of the second-messenger systems in the rat spinal cord during prolonged peripheral inflammation. Pain 58: 63-75.

Jankowska, E., Rasta, J. and Zarzecki, P. (1979). Segmental and supraspinal input to cells of origin of non-primary fibres in the feline dorsal columns. J. Physiol. 290: 185-200.

Jessell, T., Tsunoo, A., Kanazawa, I. and Otsuka, M. (1979). Substance P: depletion in the dorsal horn of rat spinal cord after section of the peripheral processes of primary sensory neurons. Brain Res. 168: 247-259.

Ju, G., Hokfelt, T., Brodin, E., Fahrenkrug, J., Fischer, J.A., Frey, P., Elde, R.P. and Brown, J.C. (1987). Primary sensory neurons of the rat showing cacitonin gene-related peptide immunoreactivity and their relation to substance P, somatostatin, galanin ,vasoactive intestinal peptide and CCK-immunoractive ganglion cells. Cell Tissue Research 247: 417-431.

Kage, R., Hershey, A.D., Krause, J.E., Boyd, N.D. and Leeman, S.E. (1991). Characterisation of the substance P receptor in tunicamycin-treated transfected cells using a photoaffinity analog. Soc. Neurosci. Abstr. 17: 805.

Kamogawa, H. and Bennett, G.J. (1986). Dorsal column postsynaptic neurons in the cat are excited by myelinated nociceptors. Br. Res. 364: 386-90.

Kanazawa, I., Ogawa, T., Kimura, S. and Munekata, E. (1984). Regional distribution of substance P, neurokinin α and neurokinin β in rat central nervous system. Neuroscience Res. 2: 111-120.

Kangawa, K., Minamino, N., Kukuda, A. and Matsuo, H. (1983). Neuromedin K: A novel mammalian tachykinin identified in porcine spinal cord. Biochem. Biophys. Res. Commun. 114(2): 533-540.

Kangrga, I. and Randic, M. (1990). Tachykinins and calitonin gene-related peptide enhance release of endogenous glutamate and aspartate from the rat spinal dorsal horn slice. J. Neuroscience 10(6): 2026-2038.

Katoh, S., Hisano, S., Kawano, H., Kagotani, Y. and Daikoku, S. (1988). Light and electron -microscopic evidence of co-storing of immunoreactive enkephalins and substance P in dorsal horn neurons of rat. Cell Tissue Res. 253: 297-303.

Kehl, L.J., Gogas, K.R., Lichtblau, L., Pollock, C.H., Mayes, M., Basbaum, A.I. and Wilcox, G.L. (1991). The NMDA antagonist MK-801 reduces noxious stimulus-evoked Fos expression in the spinal dorsal horn. In: M.R. Bond, J.E. Charlton and C.J. Woolf (Eds). Pain Research and Clinical Management, Vol. 4, Proc. VIth World Congress on Pain, Elsevier, Amsterdam, 307-311.

Kellstein, D.E., Price, D.D., Hayes, R.L. and Mayer, D.J. (1990). Evidence that substance P selectively modulates C-fibre evoked discharges of dorsal horn nociceptive neurons. Br. Res. 526: 291-298.

Kenshalo, D.R., Leonard, R.B., Chung, J.M. and Willis, W.D. (1979). Responses of primate spinothalamic neurons to graded and repeated noxious heat stimuli. J. Neurophysiol. 42(5): 1370-89.

Kenshalo, D.R., Leonard, R.B., Chung, J.M. and Willis, W.D. (1982). Facilitation of the responses of primate spinothalamic cells to cold and to tactile stimuli by noxious heating of the skin. Pain 12: 141-152.

Kenshalo, D.R., Anton, F. and Dubner, R. (1989). The detection and perceived intensity of noxious thermal stimuli in monkey and human. J. Neurophysiol. 62(2): 429-436.

Kevetter, G.A. and Willis, W.D. (1983). Collaterals of spinothalamic cells in the rat. J. Comp. Neurol. 215: 453-464.

Kevetter, G.A., Haber, L.H., Yezierski, R.P., Ching, J.M., Martin, R.F. and Willis, W.D. (1982). Cells of origin of the spinoreticular tract in the monkey. J. Comp. Neurol. 207: 61-74.

Kimura, S., Okada, M., Sugita, Y., Kanazawa, I. and Munekata, E. (1983). Novel neuropeptides: neurokinin α and β isolated from porcine spinal cord. Proc. Jpn. Acad. 59: 101-104.

Kiyama, H. and Emson, P.C. (1991). Colchicine-induced expression of proneurotensin mRNA in rat striatum and hypothalamus. Molec. Brain Res. 9: 353-358.

Kotani, H., Hoshimaru, M., Nawa, H. and Nakanishi, S. (1986). Structure and gene organisation of bovine neuromedin K precursor. Proc. Natl. Acad. Sci. (USA). 83: 7074-78.

Krause, J.E., Chirgwin, J.M., Carter, M.S., Xu, Z.S. and Hershey, A.D. (1987). Three rat preprotachykinin mRNAs encode the neuropeptides substance P and neurokinin A. Proc. Natl. Acad. Sci. (USA). 84: 881-885.

Kress, M., Koltzenburg, M., Reeh, P.W. and Handwerker, H.O. (1992). Responsiveness and functional attributes of electrically localised terminals of cutaneous C-fibres *in vitro* and *in vivo*. J. Neuropharmacology 54(2): 581-595.

Kumazawa, T. and Perl, E.R. (1977). Primate cutaneous sensory units with unmyelinated (C) afferent fibres. J. Neurophysiol. 40: 1325-1338.

Kuraishi, Y., Hirota, N., Sato, Y., Hanishama, N., Takagi, H. and Satoh, M. (1989). Stimulus specificity of peripherally-evoked substance P release form the rabbit dorsal horn in situ. Neuroscience 30: 241-250.

Laird, J.M.A., Hargreaves, R.J. and Hill, R.G. (1993). The effect of RP 67580, a non-peptide neurokinin receptor antagonist, on facilitation of a nociceptive spinal flexion reflex in the rat. Br. J. Pharmacol. 109: 713-718.

LaMotte, R.H. (1977). Distribution of the tract of Lissuauer and the dorsal root fibres in the primate spinal cord. J. Comp. Neurol. 172: 529-562.

LaMotte, R.H. and Campbell, J.N. (1978). Comparison of warm and nociceptive C-fibre afferents in monkey with human judgements of thermal pain. J. Neurophysiol. 41: 509-528.

LaMotte, R.H., Thalhammer, J.G. and Robinson, C.J. (1983). Peripheral neural correlates of magnitude of cutaneous pain and hyperalgesia: A comparison of neural events in monkey with sensory judgements in human. J. Neurophysiol. 50: 1-26.

LaMotte, R.H., Lundberg, L.E.R. and Torebjork, H.E. (1992). Pain, hyperalgesia and activity in nociceptive C units in humans after intradermal injection of capsaicin. J. Physiol. 447: 149-169.

Laneuville, O., Dorais, J. and Couture, R. (1988). Characterisation of the effects produced by neurokinins and three agonists selective for neurokinin receptor subtypes in a spinal nociceptive reflex of the rat. Life Sci. 42: 295-1305.

Lang, C.W., Duggan, A.W. and Hope, P.J. (1991). Analgesic doses of morphine do not reduce noxious evoked release of immunoreactive neurokinins in the dorsal horn of the spinal cord. Br. J. Pharm. 103: 1871-1876.

Lawson, S.N., Crepps, B.A., Bao, J., Brighton, B.W. and Perl, E.R. (1994). Substance P-like immunoreactivity (SP-LI) in dorsal root ganglia (drgs) of anaesthetised guinea-pigs is related to sensory receptor type in A- and C- fibre neurons. J. Physiol. 476: 39P.

Lazarus, L.H., Linnoila, R.I., Hernandez, O.and Di Augustine, R.P. (1980). A neuropeptide in mammalian tissues with physalaemin-like immunoreactivity. Nature 287: 555-558.

Leach, K.L., James, M.L. and Blumberg, P.M. (1983). Characterisation of a specific phorbol ester aporeceptor in mouse brain cytosol. Proc. Natl. Acad. Sci. (USA) 80: 4208.

Leah, J.D., Cameron, A.A., Kelly, W.D. and Snow, P.J. (1985). Co-existence of peptide immunoreactivity in sensory neurons of the cat. Neuroscience 16(3): 683-90.

Leah, J.D., Sandkuhler, J., Herdegen, T., Murashov, A. and Zimmermann, M. (1992). Potentiated expression of fos-protein in the rat spinal cord following bilateral noxious cutaneous stimulation. Neuroscience 48(3): 525-532.

Lecci, A., Giuliani, S., Patacchini, R., Viti, G. and Maggi, C.A. (1991). Role of NK_1 tachykinin receptors in thermonociception: effect of (±) CP-96,345, a non-peptide substance P antagonist, on the hot plate test in mice. Neuroscience Letts. 129: 299-302.

Le Graves, P., Nyberg, F., Terenius, L. and Hokfelt, T. (1985). Calcitonin gene-related peptide is a potent inhibitor of substance P degradation. Eur. J. Pharm. 115: 309-311.

Lembeck, F., Folkers, K. and Donnerer, J. (1981). Analgesic effects of antagonists to substance P. Biochem. Biophys. Res. Commun. 103: 1318.

Lembeck, F., Donnerer, J., Tsuchiya, M. and Nagahisa, A. (1992). The non-peptide tachykinin antagonist CP-96,345 is a potent inhibitor of neurogenic inflammation. Br. J. Pharmacol. 105: 527-530.

Levine, J.D., Fields, H.L. and Basbaum, A.I. (1993). Peptides and the primary afferent nociceptor. J. Neurosci. 13: 2273-2286.

Lew, R., Geraghty, D.P., Drapeau, G., Regoli, D. and Burcher, E. (1990). Binding characteristics of [^{125}] Bolton-Hunter [Sar^9, Met(O_2)^{11}]substance P, a new selective radioligand for the NK_1 receptor. Eur. J. Pharmacol. 184: 97-108.

Light, A.R. and Perl, E.R. (1977). Differential termination of large diameter and small diameter primary afferent fibres in the spinal dorsal grey matter as indicated by labelling with horseradish peroxidase. Neuroscience Letts. 6: 59-63.

Light, A.R. and Perl, E.R. (1979). Re-examination of the dorsal root projection to the spinal dorsal horn including observations on the differential termination of course and fine fibres. J. Comp. Neurol. 186: 117-132.

Light, A.R., Cascale, E.J. and Menetrey, D.M. (1986). The effects of focal stimulation in nucleus raphe magnus and periaqueductal grey on intracellularly recorded neurons in spinal laminae I and II. J. Neurophysiol. 56: 555-571.

Lima, D. (1990). A spinomedullary projection terminating in the dorsal reticular nucleus of the rat. Neuroscience 34(3): 577-589.

Lima, D. and Coimbra, A. (1989). Morphological types of spinomesencephalic neuron in the marginal zone (lamina I) of the rat spinal cord, as shown after retrograde labelling with cholera toxin subunit B. J. Comp. Neurol. 279: 327-39.

Liu, R.P.C. (1983). Laminar origins of spinal projection neurons to the periaqueductal grey of the rat. Br. Res. 264: 118-122.

Ljungdahl, A., Hokfelt, T. and Nilsson, G. (1978). Distribution of substance P-like immunoreactivity in the central nervous system of the rat-I. Cell bodies and nerve terminals. Neuroscience 3: 861-943.

Lutz, E.M., Mitchell, R., Johnson, M.S. and MacEwan, D. (1993). Functional expression of $5HT_{1C}$ receptor cDNA in cos-7 cells and its influence on protein kinase C. FEBS lett. 316: 228-232.

Lynn, B. and Carpenter, S.E. (1982). Primary afferent units from the hairy skin of the rat hindlimb. Brain Res. 238: 29-43.

Lynn, B. and Shakhanbeh, J. (1988). Substance P content of the skin, neurogenic inflammation and numbers of C-fibres following capsaicin application to a cutaneous nerve in the rabbit. Neuroscience 24(3): 769-775.

McCarson, K.E. and Goldstein, B.D. (1989). Naloxone blocks the formalin-induced increase of substance P in the dorsal horn. Pain. 38: 339-345.

McCarson, K.E. and Goldstein, B.D. (1991). Release of substance P into the superficial dorsal horn following nociceptive activation of the hindpaw of the rat. Brain Res. 568: 109-115.

McCarson, K.E. and Krause, J.E. (1994). NK-1 and NK-3 type tachykinin receptor mRNA expression in the rat spinal cord dorsal horn is increased during adjuvant or formalin-induced nociception. J. Neuroscience 14(2): 712-720.

MacDermott, A.B., Mayer, M.L., Westbrook, G.L., Smith, S.J. and Barker, J.L. (1986). NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurons. Nature 321: 519-522.

McEwan, D.J. and Mitchell, R. (1991). Calcium influx through 'L'-type channels into rat anterior pituitary cells can be modulated in two ways by protein kinase C (PKC-isoform selectivity of 1,2-dioctanoyl sn-glycerol). FEBS 291: 79-93.

McKnight, A.T., Maguire, J.J., Williams, B.J., Foster, A.C., Tridgett, R. and Iversen, L.L. (1988). Pharmacological specificity of synthetic peptides as antagonists at tachykinin receptors. Regul. Peptides 22: 127.

McLean, S., Ganog, A., Seymour, P.A., Snider, P.A., Desai, M.C., Rosen, T., Bryce, D.K., Longo, K.P., Reynolds, L.S. and Robinson, G. (1993). Pharmacology of CP-99,994: a non-peptide antagonist of the tachykinin neurokinin-1 receptor. J. P.E.T. 267(1): 472-9.

McNeill, D.L., Westlund, K.N. and Coggeshall, R.E. (1989). Peptide immunoreactivity of unmyelinated primary afferent axons in rat lumbar dorsal roots. J. Histochem. Cytochem. 37: 1047-1052.

Madison, D.V., Malenka, R.C. and Nicoll, R.A. (1986). Phorbol esters block a voltage-sensitive chloride current in hippocampal cells. Nature 321: 695-697.

Maggi, C.A., Patacchini, R., Giuliani, S., Rovero, P., Dion, S., Regoli, D., Giachetti, A. and Melti, A. (1990). Competitive antagonists discriminate between NK_2 tachykinin receptor subtypes. Br. J. Pharmacol. 100: 588.

Maggi, C.A., Eglezos, A., Quartara, L., Patacchnini, R. and Giachetti, A. (1991). Heterogeneity of NK_2 tachykinin receptors in hamster and rabbit smooth muscles. Regul. Peptide 37: 85-93.

Maggi, C.A., Giuliani, S., Patacchini, R., Quartara, L., Rovero, P., Rensotti, A.R., Mozrahi, J. and Giachetti, A. (1992). Heterogeneity of tachykinin NK_2 receptors in rabbit, guinea-pig and human smooth muscles. Neuropeptides 22: 93.

Maggi, C.A., Patacchini, R., Rovero, P. and Giachetti, A. (1993). Tachykinin receptors and tachykinin receptor antagonists. J. Auton. Pharmacol. 13: 23-93.

Maggio, J.E. and Hunter, J.C. (1984). Regional distribution of kassinin-like immunoreactivity in rat central and peripheral tissues and the effect of capsaicin. Brain Res. 307: 370-373.

Maggio, J.E., Sandberg, B.E.B., Bradley, C.V., Iverson, L.L., Santikam, S., Williams, D.H., Hunter, J.C. and Hanley, M.R. (1983). Substance K: a novel tachykinin in mammalian spinal cord. J. Med Sci. 152(1): 20-21.

Malinow, R., Schulman, H. and Tsien, R.W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. Science 245: 862-865.

Malmberg, A.B. and Yaksh, T.L. (1994). Voltage-sensitive calcium channels in spinal nociceptive processing: blockade of N- and P-type channels inhibits formalin-induced nociception. J. Neurosci. 14: 4882-4890.

Mantyh, P.W. and Hunt, S.P. (1985). The autoradiographic localisation of substance P receptors in the rat and bovine spinal cord and the rat and cat spinal trigeminal nucleus pars caudalis and the effect of neonatal capsaicin. Brain Res. 332: 315-324.

Mantyh, P.W., Maggio, J.E. and Hunt, S.P. (1984a). The autoradiographic distribution of kassinin and substance K binding sites is different from the

distribution of substance P binding sites in rat brain. Eur. J. Pharmacol. 102: 361-364.

Mantyh, P.W., Pinnock, R.D., Downes, C.P., Goedert, M. and Hunt, S.P. (1984b). Correlation between inositol phospholipid hydrolysis and substance P receptors in rat CNS. Nature 309: 795-797.

Mantyh, P.W., Gates, T., Mantyh, C.R. and Maggio, J.E. (1989). Autoradiographic localisation and characterisation of tachykinin receptor binding sites in the rat brain and peripheral tissue. J. Neurosci. 9(1) 258-279.

Mao, J., Mayer, D.J., Hayes, R.L. and Price, D.D. (1993). Spacial patterns of increased spinal cord membrane-bound protein kinase C and their relation to increases in ^{14}C-2-Deoxyglucose metabolic activity in rats with painful peripheral neuropathy. J. Neurophysiol. 70(2): P470-481

Martin, C.A.E., Emonds-Alt, X. and Advenier, C. (1993). Inhibition of cholinergic neurotransmission in isolated guinea-pig bronchi by SR 48968. Eur. J. Pharm. 243: 309-312.

Masu, Y., Nakayama, K., Tomaki, H., Haroda, Y., Kuno, M. and Nakanishi, S. (1987). cDNA cloning of bovine substance K through oocyte expression system. Nature 329: 836-838.

Maunz, R.A., Pitts, N.G. and Peterson, B.W. (1978). Cat spinoreticular neurons: locations, responses and changes in responses during repetitive stimulation. Brain Res. 148: 365-79.

Mayer, D.J., Price, D.D. and Becker, D.P. (1975). Neurophysiological characterisation of the anterolateral spinal cord neurons contributing to pain perception in man. Pain 1: 51-58.

Meberg, P.J., Barnes, C.A., McNaughton, B.L. and Routtenberg, A. (1993). Protein kinase C and F1/GAP-43 gene expression in hippocampus inversely related to synaptic enhancement lasting 3 days. PNAS (USA) 90: 12050-12054.

Meller, S.T., Gebhart, G.F., Maves, T.J. (1992): Neonatal capsaicin treatment attenuates spinal fos activation and dynorphin gene expression following peripheral tissue inflammation and hyperalgesia. J. Neurosci. 12(5): 1716-1725.

Melzack, R. and Wall P.D. (1965). Pain mechanisms: a new theory. Science 150(3699): 971-979.

Mendell, L. (1966). Physiological properties of unmyelinated fibre projection to the spinal cord. Exp. Neurol. 16: 316-332.

Mendell, L.M. and Wall, P.D. (1965). Response of single dorsal horn cells to peripheral cutaneous unmyelinated fibres. Nature 206: 97-99.

Menetrey, D., Giesler, G.J. Jnr. and Besson, J.M. (1977). An analysis of response properties of spinal cord dorsal horn neurons to non-noxious and noxious stimuli in the spinal rat. Exp. Brain. Res. 27: 15-33.

Menetrey, D., Chaouch, A. and Besson, J.M. (1980). Location and properties of dorsal horn neurons at the origin of the spinoreticular tract in lumbar enlargement of the rat. J. Neurophysiol. 44: 862-877.

Menetrey, D., Chaouch, A, Binder, D. and Besson, J.M. (1982). The origin of the spinothalamic tract in the rat : An anatomical study using retrograde transport of horseradish peroxidase. J. Comp. Neurol. 206: 193-207.

Menetrey, D., Gannon, A., Levine, J.D. and Basbaum, A.I. (1989). Expression of c-fos protein in interneurons and projection neurons of the rat spinal cord in response to noxious somatic, articular and visceral stimulation. J. Comp. Neurol. 285: 177-195.

Mense, S. (1990). Structure-function relationships in identified afferent neurons. Anat. Embryol. 181: 1-17.

Meringhi, A., Polak, J.M., Gibson, S.J., Gulbenkian, S., Valentino, K.L. and Peirone, S.M. (1988). Ultrastructural studies on calcitonin gene-related peptide-, tachykinins- and somatostatin- immunoreactive neurons in rat dorsal root ganglia: Evidence for the co-localisation of different peptides in single secretory granules. Cell Tissue Res. 254: 101-109.

Merlie, J.P., Isenberg, K.E., Russell, S.D. and Sanes, J.R. (1984). Denervation supersensitivity in skeletal muscle analysis with a cloned cDNA probe. J. Cell Biol. 99: 332-335.

Minami, M., Yasushi, K., Kawamura, M., Yamaguchi, T., Masu, Y., Nakanishi, S. and Satoh, M., (1989). Enhancement of preprotachykinin A gene expression by adjuvant-induced inflammation in the rat spinal cord: possible involvement of substance P-containing spinal neurons in nociception. Neuroscience Letts. 98: 105-110.

Minamino, N., Kangawa, K., Fukuda, A. and Matsuo, H. (1984a). Neuromedin L: a novel mammalian tachykinin identified in porcine spinal cord. Neuropeptides 4: 157-164.

Minamino, N., Matsuda, H., Kangawa, K. and Matsuo, H. (1984b). Regional distribution of neuromedin K and neuromedin L in rat brain and spinal cord. Biochem. Biophys. Res. Commun. 124(3).

Miranda, H.F., Bustamante, D., Kramer, V., Pelissier, T., Saavendra, H., Paelie, C., Fernandez, E. and Pinardi, G. (1992). Antinociceptive effects of Ca^{2+} channel blockers. Eur. J. Pharmacol. 217: 137.

Mjellum-Joly, N., Lund, A., Berge, O-G. and Hole, K. (1992). Intrathecal co-administration of substance P and NMDA augments nociceptive responses in the formalin test. Pain. 51: 195-198.

Mizuno, N., Nakano, K., Imaisumi, M. and Okamoto, M. (1967). The lateral cervical nucleus of the Japanese monkey (*macaca fuscala*). J. Comp. Neurol. 129: 375-384.

Mochley-Rosen, D., Basbaum, A.I. and Koshland, D.E. Jnr. (1987). Distinct cellular and regional localisation of immunoreactive protein kinase C in rat brain. Proc. Natl. Acad. Sci. (USA) 84: 4660-4664.

Mokha, S.S., McMillan, J.A. and Iggo, A. (1985). Descending control of spinal nociceptive transmission. Actions produced on spinal multireceptive neurons from the nuclei locus coerulus (LC) and raphe magnus (NRM). Exp. Brain Res. 58: 213-226.

Mokha, S.S., McMillan, J.A. and Iggo, A. (1986). Pathways mediating descending control of spinal nociceptive transmission from the nuclei locus coerulus (LC) and raphe magnus (NRM) in the cat. Exp. Brain Res. 61: 597-606.

Molander, C. and Grant, G. (1986). Laminar distribution and somatotopic organisation of primary afferent fibres from hindlimb nerves in the dorsal horn. A study by transganglionic transport of horseradish peroxidase in the rat. Neuroscience 19(1): 297-312.

Molander, C., Xu, Q. and Grant, G. (1984). The cytoarchitectonic organisation of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord. J. Comp. Neurol. 230: 133-141.

Morgan, J.I. and Curran, T. (1986). Role of ion flux in the control of c-fos expression. Nature 322: 552-555.

Morgan, J.I. and Curran, T. (1989). Stimulus transcription coupling in neurons: role of cellular immediate early genes. Trends. Neurosci. 12: 459-462.

Morin, F. (1955). A new spinal pathway for cutaneous impulses. Amer. J. Physiol. 183: 245-252.

Morton, C.R., Johnson, S.M. and Duggan, A.W. (1983). Lateral reticular regions and the descending control of dorsal horn neurons of the cat: selective inhibition by electrical stimulation. Brain Res. 275: 13-21.

Morton, C.R., Duggan, A.W. and Zhao, Z.Q. (1984). The effect of lesions of medullary midline and lateral reticular areas on inhibition in the dorsal horn produced by PAG stimulation in the cat. Br. Res. 301: 121-130.

Moussaoui, S.M., Le Prado, N., Bonici, B., Faucher, D.C., Cuine, F., Laudron, P.M. and Garrett, C. (1992). Distribution of neurokinin B in the rat spinal cord and peripheral tissues: Comparison with neurokinin A and substance P and the effects of neonatal capsaicin treatment. Neuroscience 48(4): 969-978.

Moussaoui, S.M., Montier, F., Carruette, A., Blanchard, J.C., Laduron, P.M. and Garret, C. (1993). A non-peptide NK_1 receptor antagonist, RP 67580, inhibits neurogenic inflammation post-synaptically. Br. J. Pharmacol. 109: 259-264.

Muller, J. (1842). Elements of Physiology Vol. 2. Taylor and Walton, London.

Murase, K. and Randic, M. (1984). Actions of substance P on rat spinal dorsal horn neurons. J. Physiol.(Lond) 346: 203-217.

Murray, C.W., Cowan, A. and Larson, A.A. (1991). Neurokinin and NMDA antagonists (but not a kainic acid antagonist) are antinociceptive in the mouse formalin model. Pain 44: 179.

Nagahisa, A., Asai, R., Kanai, Y., Murase, A., Tsuchiya-Nakagaki, M., Nakagaki, T., Shieh, T-C. and Taniguchi, K. (1992). Non-specific activity of (±) CP-96,345 in models of pain and inflammation. Br. J. Pharmacol. 107: 273-275.

Nagy, J.I., Vincent, S.R., Staines, W.M.A., Reisine, T.D. and Yamamura, H.I. (1980). Neurotoxic action of capsaicin on spinal substance P neurons. Brain Res. 186: 435-444.

Nagy, I., Maggi, C.A., Dray, A., Woolf, C.J. and Urban, L. (1993). The role of neurokinin and N-Methyl-D-Aspartate receptors in synaptic transmission from capsaicin -sensitive primary afferents in the rat spinal cord in vitro. Neuroscience 52(4): 1029-1037.

Nahin, R.L. and Micevych, P.E. (1986). A long ascending pathway of enkephalin-like immunoreactive spinoreticular neurons in the rat. Neurosci. Letts. 65: 271-276.

Nakanishi, S. (1991). Mammalian tachykinin receptors. Ann. Rev. Neurosci. 14: 123-136.

Naranjo, J.R., Mellstrom, B., Achaval, M. and Sassone-Corsci, P. (1991). Molecular pathways of pain: fos/jun mediated activation of a noncanonical AP-1 site in the prodynorphin gene. Neuron 6: 607-617.

Nathan, P.W. (1976). The gate control theory of pain. a critical review. Brain 99: 123-158.

Nawa, H., Kotani, H. and Nakanishi, S. (1984). Tissue-specific generation of 2 preprotachykinin mRNAs from one gene by alternate RNA splicing. Nature 312: 729-34.

Nestler, E.J. and Greenguard, P. (1983). Protein phosphorylation in the brain. Nature 305: 583-588.

Nicolau, M., Sirois, M.G., Bui, M., Plante, G., Sirois, P. and Regoli, D. (1993). Plasma extravasation by neurokinins in conscious rats: receptor characterisation with agonists and antagonists. Can. J. Physiol. Pharmacol. 71: 217-221.

Nimmo, A.J., Carstairs, J.R., Maggi, C.A. and Morrison, F.B. (1992). Evidence for the co-existence of multiple NK_2 tachykinin receptor subtypes in rat bladder. Neuropeptides 22(1): P48.

Ninkovic, M., Beaujouan, J.C., Torrens, Y., Saffroy, M., Hall, M.D. and Glowinski, J. (1985). Differential localisation of tachykinin receptors in rat spinal cord. Eur. J. Pharmacol. 106: 463-464.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. Science 233: 305-312.

Nishizuka, Y. (1989). The family of protein kinase C for signal transduction. J. Am. Med. Assoc. 262: 1826-1833.

Noguchi, K. and Ruda, M.A. (1992). Gene regulation in an ascending nociceptive pathway: inflammation-induced increase in preprotachykinin mRNA in rat lamina I spinal projection neurons. J. Neurosci. 12(7): 2563-2572.

Noguchi, K., Morita, Y., Kiyama, H., Ono, K. and Tohyama, M. (1988). A noxious stimulus induces the preprotachykinin-A gene expression in the rat dorsal root ganglia: a quantitative study using in situ hybridisation histochemistry. Mol. Brain. Res. 4: 31-35.

Noguchi, K., Kowalski, K., Traub, R., Solodkin, A., Iadarola, M.J. and Ruda, M.A. (1991). Dynorphin expression and fos-like immunoreactivity following inflammation induced hyperalgesia are colocalised in spinal cord neurons. Mol. Br. Res. 10: 227-333.

Noguchi, K., Dubner, R. and Ruda, M.A. (1992). Preproenkephalin mRNA in spinal dorsal horn neurons is induced by peripheral inflammation and is co-localised with fos and fos-related proteins. Neuroscience. 46(3): 561-570.

O'Brien, C., Woolf, C.J., Fitzgerald, M., Lindsay, R.M. and Molander, C. (1989). Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle or joint. Neuroscience 32(2): 493-502.

Ogawa, T., Kanazawa, I and Kimura, S. (1985). Regional distribution of substance P, neurokinin A and neurokinin B in rat spinal cord, nerve roots and dorsal root ganglia, the effects of dorsal root section or spinal transection. Br. Res. 359: 41-55.

Ohkubo, T., Shibata, M., Takahasi, H. and Inoki, R. (1990). Roles of substance P and somatostatin on transmission of nociceptive information induced by formalin in spinal cord. J. Pharmacol. Exp. Ther. 252: 1261-1268.

Oku, R., Satoh, M. and Takagi, H. (1987). Release of substance P in the spinal dorsal horn is enhanced in polyarthritic rats. Neurosci. Letts. 74: 315-319.

Otsuka, M. and Konishi, S. (1976). Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat. Nature 264: 83-84.

Otsuka, M. and Yangisawa, M. (1980). The effects of substance P and baclofen on motoneurons of isolated spinal cord of the newborn rat. J. Exp. Biol. 89: 201-214.

Palecek, J. Dougherty, P.M., Kim, S.H., Paleckova, V., Lekan, H., Chung, J.M., Carlton, S.M. and Willis, W.D. (1992). Responses of STT neurons to mechanical and thermal stimuli in an experimental model of peripheral neuropathy in primates. J. Neurophysiol. 68(6): 1951-66.

Palecek, J., Paleckova, V., Dougherty, P.M. and Willis, W.D. (1994). The effect of phorbol esters on the responses of promate spinothalamic neurons to mechanical and thermal stimuli. J. Neurophysiol. 71: 529-537.

Papir-Kricheli, D., Gilon, C., Chorev, M., Selinger, Z. and Devor, M. (1990). Neurochemical mediators of behavioural effects of receptor-selective substance P agonists administered intrathecally in the rat. Neuropharmacology 29(11): 1055-1065.

Parker, R.M.C., Fleetwood-Walker, S.M., Rosie, R., Munro, F.E. and Mitchell, R. (1993). Inhibition by NK_2 but not NK_1 antagonists of carrageenan-induced preprodynorphin mRNA expression in rat dorsal lamina I neurons. Neuropeptides 25: 213-222.

Patacchini, R., Astolfi, M., Quartara, L., Rovero, P., Giachetti, A. and Maggi, C.A. (1991). Further evidence for the existence of NK_2 tachykinin receptor subtypes. Br. J. Pharmacol. 104: 91-96.

Patacchini, R., Santicioti, M., Astolfi, M., Rovero, P., Viti, G. and Maggi, C.A. (1992). Activity of peptide and non-peptide antagonists at peripheral NK_1 receptors. Eur. J. Pharmacol. 215: 93.

Patterson, J.T., Coggeshall, R.E., Lee, W.T and Chung, K. (1990). Long ascending unmyelinated primary afferent fibres in the rat dorsal column: immunohistochemical localisations. Neuroscience Letts. 108: 6-10.

Perl, E.R. (1984). Characterisation of nociceptors and their activation of neurons in the superficial dorsal horn: first steps for the sensation of pain. Adv. Pain. Res. Ther. 6: 23-51.

Peschanski, M. and Besson, J.M. (1984). A spino-reticulo-thalamic pathway in the rat : an anatomical study with reference to pain transmission. Neuroscience 12(1): 165-178.

Petitet, F., Saffroy, M., Torrens, Y., Lavielle, S., Chassaing, G., Loeuillet, D., Glowinski, J. and Beaujouan, J-C. (1992). Possible existence of a new tachykinin receptor subtype in the guinea pig ileum. Peptides 13: 383-388.

Petitet, F., Beaujouan, J-C., Saffroy, M., Torrens, Y., Fardin, V. and Glowinski, J. (1993a). NK_1 tachykinin receptor in rat and guinea pig brains: pharmacological and autoradiographic evidence for a species difference. Peptides 14: 551-559.

Petitet, F., Beaujouan, J-C., Saffroy, M., Torrens, Y., Fardin, V. and Glowinski, J. (1993b). The non-peptide NK_2 antagonist SR 48968 is also a NK_3 antagonist in the guinea pig but not in the rat. Biochem. Biophys. Res. Commun. 191(1): 180-187.

Picard, P., Boucher, S., Regoli, D., Gitter, B.D., Howbert, J.J. and Couture, R. (1993). Use of non-peptide tachykinin receptor antagonists to substantiate the involvement of NK_1 and NK_2 receptors in a spinal nociceptive reflex in the rat. Eur. J. Pharmacol. 232: 255-261.

Piercey, M.F., Dobry, P.J.K, Schroeder, L.A. and Hollister, R. (1980). Morphine does not antagonise the substance P-mediated excitation of dorsal horn neurons. Brain Res. 186: 421-434.

Piercey, M.F., Schroeder, L.A., Folkers, K., Xu, J-C and Horig, J. (1981). Sensory and motor functions of spinal substance P. Science 214: 1361-1363.

Pommery de, J., Roudier, F. and Menetrey, D. (1984). Postsynaptic fibres reaching the dorsal column nuclei in the rat. Neurosci. Letts. 50: 319-23.

Post, C. and Folkers, K. (1985). Behavioural and antinociceptive effects of intrathecally injected substance P analogues in mice. Eur. J. Pharmacol. 113: 3335-343.

Pretel, S. and Piekut, D.T. (1991). Enkephalin, substance P and serotonin axonal input to c-fos-like immunoreactive neurons of the rat spinal cord. Peptides 12: 1243-1250.

Presley, R.W., Menetrey, D., Levine, J.D. and Basbaum, A.I. (1990). Systemic morphine suppresses noxious stimulus-evoked fos protein-like immunoreactivity in the rat spinal cord. J. Neurosci. 10(1): 323-335.

Price, D.D. and Dubner, R. (1977). Neurons subserve the sensory discriminative aspects of pain. Pain 3: 307-38.

Price, D.D. and Mayer, D.J. (1975). Neurophysiological characterisation of the anterolateral quadrant neurons subserving pain in M. Mulatta. Pain 1: 59-72.

Price, D.D., Dubner, R. and Hu, J.W. (1976). Trigeminothalamic neurons in nucleus caudalis responsive to tactile and nociceptive stimulation of monkey's face. J. Neurophysiol. 39: 936-953.

Price, D.D., Hayes, R.L., Ruda, M.A. and Dubner, R. (1978). Spatial and temporal transformations of input to spinothalamic tract neurons and their relation to somatic sensation. J. Neurophysiol. 41: 933-947.

Price, D.D., Hayashi, H., Dubner, R. and Ruda, M.A. (1979). Functional relationships between neurons of marginal and substantia gelatinosa layers of primate dorsal horn. J. Neurophysiol. 42: 1590-1608.

Proshanky, E. and Egger, M.D. (1977). Staining of the dorsal root projection to the cat's dorsal horn by anterograde movement of horseradish peroxidase. Neuroscience Letts. 5: 103-110.

Quirion, R. and Dam, T.V. (1985). Multiple tachykinin receptors in guinea pig brain. High densities of substance K (neurokinin A) binding sites in substantia nigra. Neuropeptides 6: 191-204.

Quirion, R. and Dam, T-V. (1988). Multiple tachykinin receptors: recent developments. Regul. Peptides 22: 18-25.

Radhakrishnan, V. and Henry, J.L. (1991). Novel substance P antagonist, CP-96,345 blocks responses of cat spinal dorsal horn neurons to noxious cutaneous stimulation and to substance P. Neurosci. Letts. 132: 39-43.

Ralston, H.J. and Ralston, D.D. (1979). The distribution of dorsal root axons in laminae I, II and III of the Macaque spinal cord: a quantitative electron microscope study. J. Comp. Neurol. (1979). 184: 643-84.

Ramon y Cajal, S. (1909). Histologie du systeme nerveux de l'homme et des vertebres. Vol. I pp 908-911. Inst. Cajal Madrid. Reprinted in 1952. Cited from Fitzgerald (1984).

Randic, M. and Miletic, C.V. (1977). Effect of substance P on cat dorsal horn neurons activated by noxious stimuli. Brain Res. 128: 164-169.

Rang, H.P. and Ritchie, J.M. (1988). Depolarisation of unmyelinated fibres of the rat vagus nerve produced by activation of protein kinase C. J. Neurosci. 8(7): 2606-2617.

Regoli, D., Drapeau, G., Dion, S. and D'Orleans-Juste, P. (1987). Pharmacological receptors for substance P and neurokinins. Life Sci. 40: 109.

Regoli, D., D'Orleans-Juste, P., Dion, S., Rhaleb, N-E., Rouissi, N. and Drapeau, G. (1988). Neurokinin selective agonists: discovery and pharmacological applications. Regul. Peptides 22: 153.

Rethelyi, M. (1977). Pre-terminal and terminal axon arborizations in the substantia gelatinosa of the cat's spinal cord. J. Comp. Neurol. 172: 511-528.

Retheyli, M. and Szentagothai, J. (1973). Distribution and connections of afferent fibres in the spinal cord. In : Handbook of Sensory Physiology II : Somatosensory System. A. Iggo (Ed). Springer-Verlag, Berlin. P 208-252.

Rexed, B. (1952). The cytoarchitectonic organisation of the spinal cord in the cat. J. Comp. Neurol. 96: 415-496.

Rexed, B. (1954). A cytoarchitectonic atlas of the spinal cord in the cat. J. Comp Neurol 100: 297-380.

Reymann, K.G., Frey, V., Jork, R. and Mathews, H. (1988). Polymaxin B, an inhibitor of protein kinase C, prevents the maintenance of synaptic long-term potentiation in hippocampal CA1 neurons. Brain Res. 440: 305-314.

Rolando, L. (1824). Ricerche anatomiche sulla struttura del midollo spinale. Torino: Dalla Stamperia Reale p60 (Cited by Cervero and Iggo (1980)).

Rosecrans, J.A. and Chance, W.T. (1976). Emotionally-induced antinociception. Soc. Neurosci. Abstr. 2: 919.

Ruda, M.A., Cohen, L., Shiosaka, S., Takahashi, O., Allen, B., Humphrey, E. and Iadarola, M.J. (1989). In situ hybridisation histochemical and immunohistochemical analysis of opioid gene products in a rat model of peripheral inflammation. From: Processing of sensory information in the superficial dorsal horn of the spinal cord. F. Cervero, G.J. Bennett and P.M. Headley (Eds). Plenum Publishing Corporation 1989.

Rupniak, N.M.J., Boyce, S., Williams, A.R., Cook, G., Longmore, J., Seabrook, G.R., Caeser, M., Iversen, S.D. and Hill, R.G. (1993). Antinociceptive activity of NK_1 receptor antagonists. Non specific effects of racemic RP 67580. Br. J. Pharmacol. 110: 1607-1613.

Rusin, K.I., Ryu, P.D. and Randic, M. (1992). Modulation of excitatory amino acid responses in rat dorsal horn neurons by tachykinins. J. Neurophysiol 68(1): 265-285.

Rusin, K.I., Bleakman, D., Chard, P.S., Randic, M. and Miller, R.J. (1993). Tachykinins potentiate N-methyl-D-aspartate responses in acutely isolated neurons from dorsal horn. J. Neurochemistry 60(3):952-960.

Russo, R.E. and Hounsguaard, J. (1994). Short-term plasticity in turtle dorsal horn neurons mediated by L-type Ca^{2+} channels. Neurosci. 61: 191-197.

Rustioni, A. and Kaufman, A.B. (1977). Identification of cells of origin of non-primary afferents to the dorsal column nuclei of the cat. Exp. Brain Res. 27: 1-14.

Ryall, R.W. and Pini, A. (1987). A depressant action of substance P on spinal nociceptive neurons in the rat and cat. In J.L. Henry, R. Couture, A.C. Cuello, G. Pelletier, R. Quirion and D. Regoli (Eds). Substance P and Neurokinins. Springer, New York P239-240.

Saffroy, M., Beaujouan, J.C., Torrens, Y., Besseyre, J., Bergstrom, L. and Glowinski, J. (1988). Localisation of tachykinin binding sites (NK_1,NK_2 and NK_3 ligands) in the rat brain. Peptides 9: 227-241.

Sakurada, T., Tan-No, K., Yamada, T., Sakurada, S., Kisara, K., Ohba, M. and Terenius, L. (1991). N-terminal substance P fragments inhibit the spinally induced NK_1 receptor-mediated behavioural responses in mice. Life Sci./Pharmacol. Letts. 47: PL109-PL113.

Salt, T.E. and Hill, R.G. (1983). Neurotransmitter candidates of somatosensory primary afferent fibres. Neuroscience. 10(4): 1083-1103.

Salter, M.W. and Henry, J.L. (1991). Responses of functionally identified neurons in the dorsal horn of the cat spinal cord to substance P, neurokinin A and physalaemin. Neuroscience 43(2/3): 601-610.

Sandkhuler, J., Helmchen, C., Fu, Q.G. and Zimmermann, M. (1988). Inhibition of spinal nociceptive neurons by excitation of cell bodies or fibres of passage at various brainstem sites in the cat. Neurosci. Letts. 93: 67-72.

Saria, A., Gamse, R., Petermann, J., Fischer, J.A., Theodorrson-Norheim, E. and Lundberg, J.M. (1986). Simultaneous release of several tachykinins and calcitonin gene-related peptide from rat spinal cord slices. Neurosci. letts. 63: 310-314.

Sasai, Y. and Nakanishi, S. (1989). Molecular characterisation of rat substance K receptor and its mRNAs. Biochem. Biophys. Res. Commun. 165(2): 695-702.

Sastry, B.R. (1978). Antagonism of substance P induced facilitation of central neuronal activity by 2,4-dinitrophenol and depressent agents. Can. J. Physiol. Pharmacol. 66: 527-530.

Sastry, B.R. (1979). Substance P effects on spinal nociceptive neurons. Life Sci. 24: 2169-2178.

Schafer, M.K-H., Hohr, D., Krause, J.E., Weihe, E. (1993). Inflammation-induced upregulation of NK_1 receptor mRNA in dorsal horn neurons. Neuroreport 4(8): 1007-1010.

Schaible, H-G, Jarrott, B., Hope, P.J. and Duggan, A.W. (1990). Release of immunoreactive substance P in the spinal cord during development of acute arthritis in the knee joint of the cat: a study with antibody microprobes. Brain Res. 529: 214-223.

Scheibel, M.E. and Scheibel, A.B. (1968). Terminal axonal patterns in cat spinal cord II. The dorsal horn. Brain Research 9: 32-58.

Schmidt, A.W., McLean, S. and Heym, J. (1992). The substance P receptor antagonist CP-96,345 interacts with calcium channels. Eur. J. Pharmacol. 215: 351-2.

Senba, E., Yanaihara, C., Yanaihara, N. and Tohyama, M. (1988). Co-localisation of substance P and Met-Enkephalin-Arg^6-Gly^7-Leu^8 in the intraspinal neurons of the rat, with special reference to the neurons in the substantia gelatinosa. Br. Res. 453: 110-116.

Sharp, F.R., Sagar, S.M., Hicks, K., Lowenstein, D. and Hisanaga, K. (1991). c-fos mRNA, fos and fos-related antigen induction by hypertonic saline and stress. J. Neuroscience. 11(8): 2321-2331.

Sharp, J.W., Sagar, S.M., Hisagna, K., Jasper, P. and Sharp, F.R. (1990). The NMDA receptor mediates cortical induction of fos and fos-related antigens following cortical injury. Exp. Neurol. 109: 323-332.

Shepheard, S.L., Williamson, D.J., Hill, R.G., Hargreaves, R.J. (1993). The non-peptide neurokinin$_1$ receptor antagonist RP 67580, blocks neurogenic extravasation in the dura mater of rats. Br. J. Pharmacol. 108: 11-12.

Sherrington, C.S. (1900). The muscular sense. In : Textbook of Physiology. P 1002-1025. E.A. Schaeffer (Ed). Pentland, Edinburgh. Cited in Burgess and Perl (1978).

Shigemoto, R., Yokota, Y., Tsuchida, K. and Nakanishi, S. (1990). Cloning and expression of rat neuromedin K receptor cDNA. J. Biol. Chem. 265: 623-628.

Shults, C.W., Yajima, H., Gullner, H-G, Chase, T.N. and O'Donohue, T.L. (1985). Demonstration and distribution of kassinin-like material (substance K) in the rat central nervous system. J. Neurochem. 45: 552-558.

Simone, D.A., Sorkin, L.S., Oh, U., Chung, J.M., Owens, C., La Motte, R.H. and Willis, W.D. (1991). Neurogenic hyperalgesia: central neural correlates in responses of spinothalamic tract neurons. J. Neurophysiol. 66: 228-246.

Sinclair, D. (1967). Cutaneous Sensation- The Major Theories. Oxford University Press. p 1-18.

Smith, G.D., Seckl, J.R. and Harmar, A.J. (1993). Distribution of neuropeptides in dorsal root ganglia of the rat; substance P. somatostatin, and calcitonin gene-related peptide. Neuroscience Letts. 153: 5-8.

Snider, R.M., Constantine, J.W., Lowe, J.A., Longo, K.P., Lebel, W.S., Woody, H.A., Drozda, S.E., Desai, M.C., Vinick, F.J., Spencer, R.W. (1991). A potent non-peptide antagonist of the substance P (NK1) receptor. Science 251: 435-437.

Song, X.J. and Zhao, Z.Q. (1994). Interaction between substance P and excitatory amino acid receptors in modulation of nociceptive responses of cat spinal dorsal horn neurons. Neurosci. Letts. 168: 49-52.

Sonnenberg, J.L., Mitchelmore, C., MacGregor-Leon, P.F., Hempstead, J., Morgan, J.I. and Curran, T. (1989). Glutamate receptor agonists increase the expression of Fos, Fra and AP-1 DNA binding activity in the mammalian brain. J. Neuroscience Res. 24: 72-80.

Stucky, C.L., Galeassa, M.T., Seybold, V.S. (1993). Time-dependent changes in bolton-hunter labelled ^{125}I-substance P binding in rat spinal cord following unilateral adjuvant-induced peripheral inflammation. Neuroscience 57(2): 397-409.

Sullivan, A.F. and Dickenson, A.H. (1991). Electrophysiologic studies on the spinal antinociceptive action of kappa opioid agonists in the adult and 21-day-old rat. J. P.E.T. 256(3): 1119-25.

Sweeney, M.I. and Sawynok, J. (1986). Evidence that substance P may be a modulator rather than a transmitter of noxious mechanical stimulation. Can. J. Physiol. Pharmacol. 64: 1324-2327.

Swett, J.E., McMahon, S.B. and Wall, P.D. (1985). Long ascending projections to the midbrain from cells of lamina I and nucleus of the dorsolateral funiculus of the rat spinal cord. J. Comp. Neurol. 238: 401-416.

Szentagothai, J. (1964). Neuronal and synaptic arrangement in the substantia gelatinosa rolandi. J. Comp. Neurol. 122 219-239.

Takeda, Y. and Krause, J.E. (1991). Pharmacological and molecular biological studies on the diversity of rat tachykinin NK_2 receptor subtypes in rat CNS, Duodenum, Vas Deferens and Urinary Bladder. Annals.New York Acad.Science 632: 479-482.

Tashiro, T. and Ruda, M.A. (1988). Immunocytochemical identification of axons containing co-existent serotonin and substance P in the cat lumbar spinal cord. Peptides 9: 383-391.

Tateishi, K., Matsuo, Y. and Hamaoka, T. (1989). Establishment of highly specific radioimmunoassays for neurokinin A and neurokinin B and determination of tissue

distribution of these peptides in rat central nervous system. Regul. Peptides 24: 245-257.

Tessler, A., Gazer, E., Artymyshyn, R., Murray, M. and Goldberger, M.E. (1980). Recovery of substance P in the cat spinal cord after unilateral lumbosacral deafferentation. Br. Res. 191: 459-470.

Thio, C.L. and Sontheimer, H. (1993). Differential modulation of TTX-sensitive and TTX-resistant Na^+ channels in spinal cord astrocytes following activation of protein kinase C. J. Neurosci. 13: 4889-4897.

Thompson, S.W.N., Urban, L. and Dray, A. (1993). Contribution of NK_1 and NK_2 receptor activation to high threshold afferent fibre evoked ventral root responses in the rat spinal cord in vitro. Br. Res. 625: 100-108.

Thompson, S.W.N., Dray, A. and Urban, L. (1994). Injury-induced plasticity of spinal reflex activity: NK_1 neurokinin receptor activation and enhanced A- and C-fibre mediated responses in the rat spinal cord in vitro. J. Neurosci. 14(6): 3672-3687.

Thomson, C., Boel, E. and Sudaz, P.D. (1994). Actions of phenylglycine analogs at subtypes of the metabotropic glutamate receptor family. Eur. J. Pharmacol. 267: 77-84

Tingley, W.G., Roche, K.W., Thompson, A.K. and Huganir, R.L.(1993). Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. Nature 364: 70-73.

Tiseo, P.J., Adler, M.W. and Liu-Chen, L-Y. (1990). Differential release of substance P and somatostatin in the rat spinal cord in response to noxious cold and heat: effect of Dynorphin $A_{(1-17)}$. J. P.E.T. 252(2): 539-545.

Tolle, T.R., Castro-Lopes, J.M., Evan, G. and Zieglzansberger, W. (1991). C-fos induction in the spinal cord following noxious stimulation: prevention by opiates but not by NMDA antagonists. In M.R. Bond, J.E. Charlton and C.J. Woolf (Eds), Pain Research and Clinical Management, Vol. 4, Proc. VIth World Congress on Pain, Elsevier, Amsterdam, 229-305.

Tolle, T.R., Ableitner, A., Castro-Lopes, J.M. and Zieglezansberger, W. (1994). C-fos protein, prodynorphin mRNA and protein kinase C are altered with distinct spacial and temporal patterns in the spinal cord of monarthritic rats. Proceedings of the 7th World Congress on Pain. Prog. in Pain Res. and Management Vol 2. G.F. Gebhart, D.L. Hammond and T.S. Jensen (Eds). IASP Press, Seattle.

Too, H.P. and Maggio, J.E. (1992). Immunocytochemical localisation of neuromedin K (neurokinin B) in rat spinal ganglia and cord. Peptides 12: 431-443.

Too, H-P., Cordona, J.L. and Maggio, J.E. (1989). Heterogeneity of tachykinin-like immunoreactive peptides in rat spinal cord and dorsal root ganglia. Peptides 10: 25-30.

Torebjork, H.E. (1974). Afferent C units responding to mechanical, thermal and chemical stimuli in human non-glabrous skin. Acta. Physiol. Scand. 92: 374-390.

Torebjork, H.E. and Hallin, R.G. (1973). Perceptual changes accompanying controlled preferential blocking of A and C fibre responses in intact human skin nerves. Exp. Brain Res. 16: 321-332.

Torebjork, H.E. and Hallin, R.G. (1974). Identification of C afferent units in intact human skin nerves. Brain Res. 67: 387-403.

Toullec, D., Pianetti, P., Coste, H., Belleverge, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Coriolle, F., Duhamel, L., Charon, D., and Kirilovsky, J. (1990). The bisindolylmaleimide GF109203X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. 266: 15771-15781.

Treede, R-D., Meyer, R.A., Raja, S.N. and Campbell, J.N. (1992). Peripheral and central mechanisms of cutaneous hyperalgesia. Prog. Neurobiol. 38: 397-421.

Truex, R.C., Taylor, M.J., Smythe, M.Q. and Gildenberg, P.L. (1965). The lateral cervical nucleus of the cat, dog and man. J. Comp. Neurol. 139: 93-104.

Tsuchida, K., Shigemoto, R., Yokota, Y. and Nakanishi, S. (1990). Tissue distribution and quantification of the mRNA for three rat tachykinin receptors. Eur. J. Biochem. 193: 751-757.

Uddenberg, N. (1968). Functional organisation of long, second-order afferents in the dorsal funiculus. Exp. Brain Res. 4: 377-382.

Urban, L. and Randic, M. (1984). Slow excitatory transmission in rat dorsal horn: possible mediation by peptides. Br. Res. 290: 336-341.

Urban, L. and Randic, M. (1989). Tachykinins modulate multiple ionic conductances in voltage-clamped rat spinal dorsal horn neurons. J. Neurophysiol. 61: 854-865.

Urban, L., Maggi, C.A., Nagy, I. and Dray, A. (1992). The selective NK_2 receptor antagonist MEN 10376 inhibits synaptic excitation of dorsal horn neurons evoked by C-fibre activation in the in vitro spinal cord. Neuropeptides 22: P68.

Urban, L., Naeem, S., Patel, I.A. and Dray, A. (1994). Tachykinin induced regulation of excitatory amino acid responses in the rat spinal cord in vitro. Neuroscience Letts. 168: 185-188.

Van Giersbergen, P.L.M., Shatzer, S.A., Henderson, A.K., Lai, J., Nakanishi, S., Yamamura, H.I. and Buck, .H. (1991). Characterisation of a tachykinin peptide NK_2 receptor transfected into murine fibroblast N82 cell. Proc. Natl. Acad. Sci.(USA). 88: 1661-1665.

Van Hees, J. and Gybels, J.M. (1972). Pain related to single afferent C fibres from human skin. Brain Res. 48 397-400.

Vaught, J.L., Rothman, R.B. and Westfall, T.C. (1982). Mu and delta receptors: their role in analgesia and in the differential effects of opioid peptides on analgesia, Life Sci. 30: 1443-1455.

Vedder, H. and Otten, U. (1991). Biosynthesis and release of tachykinins from rat sensory neurons in culture. J. Neuroscience Res. 30: 288-299.

Villanueva, L., Bouhassira, D., Bing, Z. and Le Bars, D. (1988). Convergence of heteropathic and nociceptive information onto subnucleus reticularis dorsalis neurons in the rat medulla. J. Neurophysiol. 60: 980-1009.

Von Euler, U.S. and Gaddum, J.H. (1931). An unidentified depressor substance in certain tissue extracts. J. Physiol. 72: 74-87.

Von Frey, M. (1895). The Pain Sensation. In: Classical German Contributions to Pain Research. H.O. Handwerker and K. Brune (eds). (1897). Tagblatt-Druckerei KG pp 91-131

Waldeyer, H. (1888). Das Gorilla-Rueckenmark. Acad. Wissensch. Berlin 147pp. Cited from Willis and Coggeshall (1991).

Wall, P.D. (1967). The laminar organisation of the dorsal horn and effects of descending impulses. J. Physiol. 188: 403-424.

Wall, P.D. and Woolf, C.J. (1984). Muscle but not cutaneous C-afferent input produces prolonged increases in the excitability of the flexion reflex in the rat. J. Physiol. 356: 443-458.

Wang, J-H. and Feng, D-P. (1992). Postsynaptic protein kinase C essential to induction and maintenance of long-term potentiation in the hippocampal CA1 region. Proc. Natl. Acad. Sci.(USA) 89: 2576-2580.

Warden, K. and Young, W.S. (1988). Distribution of cells containing mRNAs encoding substance P and neurokinin B in the rat central nervous system. J. Comp. Neurol. 272: 90-113.

Watling, K.J., Guard, S., Howsen, W., and Walton, L. (1991). Inhibition of binding to tachykinin NK_1 receptor sites in rat, guinea pig and human brain by the non-peptide substance P antagonist CP-96,345. Br. J. Pharmacol. 104: 27P.

Watling, K.J., Guard, S., Boyle, S.J., McKnight, A.T. and Woodruff, C.N. (1994). Species variants of tachykinin receptor types· Biochem. Soc. Trans. 22: 118-122.

Weihe, E., Nohr, D., Millan, M.J., Stein, C. and Muller, S. (1988). Peptide neuroanatomy of adjuvant-induced arthritic inflammation in rat. Agents and Actions 25: 233-239.

White, J.C. and Sweet, W.H. (1969). Pain and the neurosurgeon. Springfield IL : Thomas (1969). Cited from Smith *et al* (1991).

White, J.D. and Gall, C. (1987). Differential regulation of neuropeptide and protooncogenic mRNA content in the hippocampus following recurrent seizures. Mol. Brain Res. 427: 21-29.

Wiberg, M. and Blomqvist, A. (1984). The spinomesencephalic tract in the cat : its cells of origin and termination pattern as demonstrated by the intraaxonal transport method. Br. Res. 291: 1-18.

Wiesenfeld-Hallin, Z. (1986). Substance P and somatostatin modulate spinal cord excitability via physiologically different sensory pathways. Brain Res. 372: 172-175.

Wiesenfeld-Hallin, Z., Xu, X-J., Hakanson, R., Feng, D.M. and Folkers, K. (1990). The specific antagonist effect of intrathecal spantide II on substance P and C fibre conditioning stimulation-induced facilitation of the nociceptive flexor reflex in the rat. Br. Res. 526: 284-290.

Wiesenfeld-Hallin, Z., Luo, L., Xu, X-J. and Maggi, C.A. (1994). Differential effects of selective tachykinin NK_2 receptor antagonists in rat spinal cord. Eur. J. Pharmacol. 251: 99-102.

Willcockson, W.S., Chung, J.M., Hori, V., Leek, H. and Willis, W.D. (1984). Effects of iontophoretically-released peptides on primate spinothalamic cells. J. Neurosci. 4: 741-750.

Williams, B.T., Curtis, N.R., McKnight, A.T., Maguire, J., Foster, A. and Tridgett R. (1988). Development of NK_2 selective antagonists. Regul. Peptides 22: 189.

Williams, S., Pini, A., Evan, G. and Hunt, S.P. (1989). Molecular events in the spinal cord following sensory stimulation. Processing of sensory information in the superficial dorsal horn. (Eds. F. Cevero, G.J. Bennett and P.M. Headley. Plenum Press.

Williams, S., Evan, G. and Hunt, S.P. (1990). Spinal c-fos induction by sensory stimulation in neonatal rats. Neurosci Letts 109(3): 309-314.

Willis, W.D. Jr. and Coggeshall, R.E. (1991). Sensory mechanisms of the spinal cord. Plenum Press. New York and London.

Willis, W.D. Jr, Trevino, D.L., Coulter, J.D. and Maunz, R.A. (1974). Responses of primate spinothalamic tract neurons to natural stimulation of the hindlimb. J. Neurophysiol. 37: 358-72.

Willis, W.D., Kenshalo, D.R. and Leonard, R.B. (1979). The cells of origin of the primate spinothalamic tract. J. Comp. Neurol. 188: 543-74.

Womack, M.D., MacDermott, A.B. and Jessell, T-M. (1988). Sensory transmitters regulate intracellular calcium in dorsal horn neurons. Nature 334: 351-353.

Womack, M.D., MacDermott, A.B. and Jessell, T-M., (1989). Substance P increases $[Ca^{2+}]_i$ in dorsal horn neurons via two distinct mechanisms. Soc. Neurosci. Abstract 15: 184.

Woolf, C.J. (1983). Evidence for a central component of post-injury pain hypersensitivity. Nature 306: 686-688.

Woolf, C.J. (1991). Generation of acute pain: central mechanisms. Br. Medical Bulletin 47(3): 523-533.

Woolf, C.J. and King, A.E. (1990). Dynamic alterations in the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat spinal cord. J. Neuroscience 10(8): 2717-2726.

Woolf, C.J. and Thompson, S.W.N. (1991). The induction and maintenance of central senstization is dependent on N-methyl-D-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. Pain 44: 293-299.

Woolf, C.J. and Wall, P.D. (1986). The relative effectiveness of C primary afferent fibres of different origins in evoking a prolonged facilitation of the flexor reflex in the rat. J. Neuroscience 6: 1433-1443.

Woolf, C.J. and Wiesenfeld-Hallin, Z. (1986). Substance P and calcitonin gene related peptide synergistically modulated the gain of the nociceptive flexor withdrawal reflex in the rat. Neuroscience Letts 66: 226-230.

Woolfe, G. and MacDonald, A.D. (1944). Evaluation of analgesic action of pethidine hydrochloride (Demorol). J. Pharmacol. Exp. Ther. 80: 300-307.

Worley, P.F., Barbaran, I.M. and Snyder, S.H. (1986). Heterogeneous localisation of protein kinase C in rat brain: autoradiographic analysis of phorbol ester receptor binding. J. Neurosci. 6: 199-207.

Wright, D.M. and Roberts, M.H.T. (1980). Response of spinal neurons to a substance P analogue, noxious pinch and bradykinin. Eur. J. Pharmacol. 64: 165-167.

Xu, X-J. and Wiesenfeld-Hallin, Z. (1992). Intrathecal neurokinin A facilitates the spinal nociceptive flexor reflex evoked by thermal and mechanical stimuli and synergistically interacts with substance P. Acta Physiol. Scand. 44: 163-168.

Xu, X-J., Maggi, C.A. and Wiesenfeld-Hallin Z. (1991). On the role of NK_2 tachykinin receptors in the mediation of spinal reflex excitability in the rat. Neuroscience 44(2): 483-490.

Xu, X-J., Dalsguaard, C.J., Wiesenfeld-Hallin, Z. (1992a). Intrathecal CP-96,345 blocks reflex facilitation in rats induced by substance P and C-fibre conditioning stimulation. Eur. J. Pharmacol. 216: 337-344.

Xu, X-J., Dalsguaard, C-J., Maggi, C.A. and Wiesenfeld-Hallin, Z. (1992b). NK-1 but not NK-2, tachykinin receptors mediated plasma extravasation induced by antidromic C-fibre stimulation in rat hindpaw: demonstrated with the NK-1 antagonist CP-96,345 and the NK-2 antagonist Men 10207. Neuroscience Letts. 139: 249-252.

Yaksh. T.L., Jessell, T.M., Gamse, R., Mudge, A.W. and Leeman, S.E. (1980). Intrathecal morphine inhibits substance P release from mammalian spinal cord *in vivo*. Nature 286: 155-156.

Yamamoto, T. and Yaksh, T.L. (1991). Stereospecific effects of a nociceptive NK_1 selective antagonist CP-96,345: antinociception in the absence of motor dysfunciton. Life Sci. 49: 1953-1963.

Yamamoto, T. and Yaksh, T.L. (1992). Effects of intrathecal capsaicin and an NK-1 antagonist, CP-96,345 on the thermal hyperalgesia observed following unilateral constriction of the sciatic nerve in the rat. Pain 51: 329-334.

Yashpal, K. and Coderre, T.J. (1993). Contribution of nitric oxide, arachadonic acid and protein kinase C to persistent pain following tissue injury in rats. 7th World Congress on Pain Abstracts. IASP Publications, Seattle p223.

Yashpal, K., Wright, D.M. and Henry, J.L. (1982). Substance P reduces tail-flick latency: implications for chronic pain syndromes. Pain 14: 155-167.

Yashpal, K., Dam, T-V. and Quirion, R. (1990). Quantitative autoradiographic distribution of multiple neurokinin binding sites in rat spinal cord. Brain Res. 506: 259-266.

Yashpal, K., Dam, T-V. and Quirion, R. (1991). Effects of dorsal rhizotomy on neurokinin receptor subtypes in the rat spinal cord: a quantitative autoradiographic study. Brain Res. 552: 240-247.

Yashpal, K., Radhakrishnan, V., Coderre, T.J. and Henry, J.L. (1993). CP-96,345, but not its sterioisomer, CP 96,344, blocks the nociceptive responses to intrathecally administered substance P and to noxious thermal and chemical stimuli in the rat. Neuroscience 52(4): 1039-1047.

Yezierski, R.P. and Mendez, C.M. (1991). Spinal distribution and collateral projections of rat spinomesencephalic tract cells. Neuroscience 44(1): 113-130.

Yezierski, R.P. and Schwartz (1986). Response and receptive field properties of spinomesencephalic tract cells in the cat. J. Neurophysiol. 55(1): 76-96.

Yokota, Y., Sasai, Y., Tanaka, K., Fujiwara, T., Tsuchida, K., Shigemoto, R., Kakizuka, A., Ohkubo, H. and Nakanishi, S. (1989). Molecular characterisation of a functional cDNA for rat substance P receptor. J. Biol. Chem. 264: 17469-17652.

Yoshimura, M., Shimizu, T., Yajiri, Y., Inokuchi, H. and Nishi, S. (1993). Primary afferent-evoked slow EPSPs and responses to substance P of dorsal horn neurons in the adult rat spinal cord slices. Regulatory Peptides 46: 407-409.

Young, M.R., Fleetwood-Walker, S.M., Mitchell, R. and Munro, F.E. (1994). Evidence for a role of metabotropic glutamate receptors in sustained nociceptive inputs to rat dorsal horn neurons. Neuropharmacology 33(1): 141-144.

Young, M.R., Fleetwood-Walker,S.M., Birch, P.J., Boundtra, C. and Bowers, J. (1995). The role of neurokinin and metabotropic glutamate receptors in thermal and mechanical paw-withdrawal responses in normal and carrageenan-treated rats. Br. J. Pharmacol. 114: 316P.

Young, S.W. III., Bonner, T.I., Brann, M.R. (1986). Mesencephalic dopamine neurons regulate the expression of neuropeptide mRNAs in the rat forebrain. PNAS (USA) 83: 9827-9831.

Zieglezansberger, W. and Tulloch, I.F. (1979). Effects of substance P on neurons in the dorsal horn of the spinal cord of the cat. Brain Res. 166: 273-282.

Zimmermann, M. (1968). Dorsal root potentials after C stimulation. Science 160: 896-898.

Zotterman, Y. (1939). Touch, pain and tickling: an electrophysiological investigation on cutaneous sensory nerves. J. Physiol. 95: 1-28.

APPENDIX:
Publications Arising From Research.

Some of the results in this Thesis have been published:-

1. Munro, F.E., Fleetwood-Walker, S.M., Parker, R.M.C. and Mitchell, R. (1993). Mustard oil-evoked activation of rat dorsal horn neurons is inhibited by NK_2 but not NK_1 receptor antagonists. British Journal of Pharmacology 109: P243.

2. Munro, F.E., Fleetwood-Walker, S.M., Parker, R.M.C. and Mitchell, R. (1993). The effects of neurokinin receptor antagonists on mustard oil-evoked activation of rat dorsal horn neurons. Neuropeptides 25: 299-305.

3. Fleetwood-Walker, S.M., Parker, R.M.C., Munro, F.E., Young, M.R. and Mitchell, R. (1993). The role of NK_1 and NK_2 receptors in nociceptive inputs to rat dorsal horn lamina I and lamina IV/V neurons. Neuropeptides 24(4): P82.

4. Fleetwood-Walker, S., Parker, R., Munro, F., Young, M. and Mitchell, R. (1993). Evidence for the involvement of NK_2 rather than NK_1 receptors in both acute and sustained nociceptive inputs to dorsal horn neurons. Congress Abstracts, XXXII Congress of the International Union of Physiological Sciences 299.6/0.

5. Munro, F.E., Young, M.R., Fleetwood-Walker, S.M., Parker, R.M.C. and Mitchell, R. (1993). Receptor and cellular mechanisms involved in mustard oil-induced activation of dorsal horn neurons. Congress Abstracts, IASP Publications, 7th World Congress on Pain 467.

6. Fleetwood-Walker, S.M., Parker, R.M.C., Munro, F., Young, M.R. and Mitchell, R. (1993). Evidence for a role of NK_2 receptors in mediating acute and sustained nociceptive inputs to dorsal horn neurons. Congress Abstracts, IASP Publications, 7th World Congress on Pain 11.

7. Fleetwood-Walker, S.M., Parker, R.M.C., Munro, F.E., Young, M.R., Hope, P.J. and Mitchell, R. (1993). Evidence for a role of NK_2 receptors in mediating brief nociceptive inputs to rat dorsal horn (laminae III-V) neurons. European Journal of Pharmacology 242: 173-181.

8. Munro, F.E., Fleetwood-Walker, S.M. and Mitchell, R. (1994). Evidence for the involvement of protein kinase C in the activation of dorsal horn neurons evoked by cutaneous application of mustard oil. Neuroscience Letters 170:199-202.

9. Fleetwood-Walker, S.M., Parker, R.M.C., Munro, F.E., Young, M.R., Hope, P.J. and Mitchell, R. (1994). The role of tachykinins and somatostatin in spinal nociceptive processing. The Journal of Pain Society 11: 22-26.

10. Munro, F.E., Young, M.R., Fleetwood-Walker, S.M., Parker, R.M.C. and Mitchell, R. (1994). Receptor and cellular mechanisms involved in mustard oil-induced activation of dorsal horn neurons. Progress in Pain Research and Management Vol. 2: Proceedings of the 7th World Congress on Pain. Eds. Gebhart, G.F., Hammond, D.L. and Jensen, T.S. P 337-346.

See overleaf.

P135 MUSTARD OIL-EVOKED ACTIVATION OF RAT DORSAL HORN NEURONS IS INHIBITED BY NK_2 BUT NOT NK_1 RECEPTOR ANTAGONISTS

F.E. Munro, S.M. Fleetwood-Walker, R.M.C. Parker & R. Mitchell[1], Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH and [1]MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ

We have previously reported that the responses of dorsal horn neurons to brief noxious thermal (but not mechanical) cutaneous stimuli are inhibited by NK-2 but not NK-1 receptor antagonists (Fleetwood-Walker *et al*, 1991). In the *in vitro* spinal cord/dorsal root preparation, neuronal activity evoked by brief application of capsaicin to the dorsal root ganglia is also inhibited by a selective NK-2 antagonist (Urban *et al*, 1992). It is not clear, however, whether NK-1 and/or NK-2 receptors are important in the sustained activation of dorsal horn neurons by prolonged noxious stimulation which results in altered sensitivity and receptive field properties of those neurons (Woolf and King, 1990). Topical cutaneous application of the chemical irritant 3-isothiocyanato-prop-1-ene (mustard oil) causes prolonged activation of C- (and briefly also Aδ-) afferents (Heapy *et al*, 1987) and brings about a state of increased responsiveness in dorsal horn neurons (Woolf & King, 1990).

Extracellular recordings were made from laminae IV/V dorsal horn neurons in segments T_{12} - L_4 of α-chloralose/urethane-anaesthetised rats, as described previously (Fleetwood-Walker *et al*, 1991). Neuronal receptive fields were located using brief innocuous and noxious stimuli and then mustard oil (5 - 20% in paraffin oil) was topically applied to a restricted area (usually about 3 cm^2) within this region. Neuronal responses to this stimulus were recorded using the central barrel of a glass microelectrode, with ionophoresis of drugs from the side barrels. The NK-1 antagonist L-668169 and the NK-2 antagonist L-659874 were prepared as 0.15 mM aqueous solutions with 0.3% dimethylformamide, pH 4.5 - 5.0 (Fleetwood-Walker *et al*, 1991). Ionophoresis of this vehicle alone had no effect. The NK 1 antagonist GR 82334 (Hagan *et al*, 1991) was prepared as a 1 mM aqueous solution, pH 4.5 - 5.0.

In virtually all multireceptive neurons tested, mustard oil evoked a large and prolonged increase in activity with firing rates of 149 ± 36 fold of background (mean ± s.e. mean) being consistently maintained. In most cases, a steady elevated firing rate was maintained for at least 30 min by repeated application of mustard oil to the same site. Drugs were tested after the mustard oil-evoked activity was stable and had been continuous for at least 1 - 2 min. In 19 out of 21 neurons tested with L-659874 (10 - 80 nA), there was a clear and maintained inhibition of mustard oil-evoked activity to 52 ± 6% of prior control (mean ± s.e. mean) with an onset delay of 0 - 120 seconds. Recovery was readily observed after terminating ionophoresis of L-659874 in nearly all cases. In about half of the cases, this inhibition began to decline after 159 ± 34 s (mean ± s.e. mean) even during continued ionophoresis. In contrast, L-668169 and GR 82334 (20 - 80 nA) rarely inhibited mustard oil-evoked activity (in 1 out of 8 and 3 out of 9 cases respectively). In 14 cells tested with both NK-2 and NK-1 antagonist, there was only one example where both were effective.

These results provide further evidence for a role of NK-2 receptors in transducing nociceptive afferent inputs to dorsal horn neurons. NK-2 receptors appear to be important not only in the acute responses of dorsal horn neurons to brief noxious thermal stimuli, but also in their sustained activation by noxious chemical stimuli.

Fleetwood-Walker, S.M., Hope, P.J., Parker, R.M.C. & Mitchell, R. (1991) Br. J. Pharmacol. **104**, 472P.
Hagan, R.M., Ireland, S.J., Bailey, F. McBride, C., Jordon, C.C. & Ward, P. (1991) Br. J. Pharmacol. **102**, 168P.
Heapy, C.G., Jamieson, A. & Russell, N.J.W. (1987) Br. J. Pharmacol. **90**, 164P.
Urban, L., Maggi, C.A., Nagy, I. & Dray, A. (1992) Neuropeptides **22**, 68.
Woolf, C.J. & King, A.E. (1990) J. Neurosci. **10**, 2717-2726.

NEUROPEPTIDES

Neuropeptides (1993) **25**, 299–305

The Effects of Neurokinin Receptor Antagonists on Mustard Oil-evoked Activation of Rat Dorsal Horn Neurons

F. E. MUNRO, S. M. FLEETWOOD-WALKER, R. M. C. PARKER and R. MITCHELL*

*Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH and *MRC Brain Metabolism Unit, University Department of Pharmacology, 1 George Square, Edinburgh EH8 9JZ (Reprint requests to SF-W)*

Abstract—Previous evidence indicated that brief nociceptive responses of neurons in laminae IV/V of both rat and cat dorsal horn are more readily inhibited by antagonists at NK_2 rather than at NK_1 neurokinin receptors. Further support for a role of spinal NK_2 receptors in nociception has been provided from experiments assessing modulation of the nociceptive flexor reflex by tachykinins and activation of dorsal horn neurons by brief application of capsaicin to afferents. The present experiments were designed to compare the contribution of NK_1 and NK_2 receptors in dorsal horn to the sustained neuronal activity induced by peripheral application of the chemical algogen mustard oil (reported to be a selective activator of C afferents).

In nearly all of the multireceptive laminae IV/V neurons tested, a selective NK_2 receptor antagonist L 659,874 inhibited previously established mustard oil-induced activity. In contrast, two selective NK_1 receptor antagonists L 668,169 and GR 82334 were only rarely effective. These results further underline the apparent importance of NK_2 receptors in spinal nociceptive processing. NK_1 receptors do not appear to play a major role in the present experimental protocol, but they may of course do so under different circumstances.

Introduction

Processing of the precursor for Substance P (SP), preprotachykinin-1 can lead to the generation of neurokinin A (NKA) and related sequences which demonstrate selectivity for the NK_2 class of neurokinin receptor.[1–4] In addition to SP, NKA is present in spinal dorsal horn, being largely associated with fine afferents arriving through the dorsal roots[5–7] and being released into the dorsal horn by noxious (but not damaging) cutaneous stimuli.[8] The vast majority of NK_2 receptors are present in peripheral non-neural tissue, but recent evidence from radioligand binding studies and from specific probe hybridisation to the NK_2 receptor mRNA has revealed very small but significant populations of NK_2 receptors in the CNS and in particular in the spinal cord.[9–11] In contrast, there is strong evidence

Date received 17 December 1992
Date revised 12 March 1993
Date accepted 10 April 1993

Correspondence to S. M. Fleetwood-Walker, Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH, UK

for a major population of NK_1 binding sites in superficial dorsal horn and for the presence of moderately high levels of NK_1 receptor mRNA in spinal cord.[9,12]

Both SP and NKA are released into superficial dorsal horn by a regularly repeated noxious pinch stimulus.[13,14] However, whilst SP was not released by noxious thermal stimuli at non-damaging levels,[13] both increasing the temperature to elicit chronic inflammatory damage and induction of experimental arthritis with intra-articular algogens do result in release of SP into dorsal horn.[14,15] Indeed it has been suggested that SP and NK_1 receptors may not be important in brief nociceptive responses but rather in their potentiation following prolonged noxious stimulation.[16]

Functional experiments in our laboratory have shown that locally-administered NK_2 but not NK_1 receptor antagonists inhibited the responses of both rat and cat dorsal horn neurons to brief noxious thermal stimuli applied to their cutaneous receptive field.[17–20] Correspondingly, NK_2 but not NK_1 receptor-selective agonists enhanced neuronal responses to these noxious stimuli without altering either basal or other stimulus-evoked activity. Although we found no evidence in our experiments for a role of dorsal horn NK_1 receptors and SP in mediating responses to brief noxious, thermal, or indeed mechanical, stimuli, many other groups have provided data to strongly implicate some such role in nociceptive processing.[16,21–31]

In view of the possibility that SP may play a more important role in responses to such prolonged nociceptive inputs we have investigated here the responses to the sustained activity in C-afferents elicited by cutaneous application of the chemical algogen mustard oil.[32]

Materials and methods

Experiments were carried out on 33 male Wistar rats (250–340 g), anaesthetised with intravenous (i.v.) α-chloralose (350 mg kg^{-1}) and urethane (700 mg kg^{-1}), after induction with halothane. Supplementary doses of α-chloralose were given as required. Core temperature was maintained at 37–38°C with a thermostatically controlled heated blanket, and in the majority of experiments carotid blood pressure was monitored throughout the experiment. Oxygen (0.1 l min^{-1}) was passed over the end of the tracheal cannula to enrich the inspired air. The thoraco-lumbar spinal column was supported by three pairs of clamps. A laminectomy (segments L1–L4) was then carried out and agar injected under the most rostrally clamped vertebra and then over the whole area of the laminectomy. A core of agar was removed from above the recording region, the dura carefully cut and a pool of liquid paraffin applied to the region.

Electrophysiological methods and ionophoresis

Extracellular recordings were made via the central barrel (4 M NaCl, pH 4.0–4.5) of a 7-barrelled glass microelectrode. Electrode tip sizes were 4.0–4.5 μm and DC resistances were 5–8 MΩ. The band-width of the recording amplifier was 1 Hz–7kHz. One side barrel contained 1M NaCl (pH 4.0–4.5) for automatic current balancing and current controls (Neurophore Ionophoresis System, Medical Systems Corporation). Another side barrel contained Pontamine Sky Blue dye (2% in 0.5 M sodium acetate for marking recording sites by ejection at 10 μA for 10 min). (The laminar locations of recorded neurons were assessed from Pontamine Sky Blue spots on 15 μm cryostat sections lightly stained with eosin). The other barrels of the electrode contained neurokinin receptor antagonists, vehicle, or 0.1 M d,l-homocysteic acid (pH 8 in H_2O) for direct testing of neuronal excitability. The antagonists L 659,874 and L 668,169[33,34] were obtained from Cambridge Research Biochemicals and prepared as 0.15 mM solutions in 0.3% dimethylformamide in water (diluted from concentrated stocks in dimethylformamide). GR 82334[35] was prepared as a 1 mM solution in water. All peptide solutions were either prepared shortly before use or stored at –20°C in aliquots. The pH of the antagonist solutions was adjusted to pH 4.5 and all were ejected using cathodal currents. Retaining currents of –10 nA were used to minimise drug leakage between tests. Action potentials of the recorded cells were clearly discriminated from other field potentials throughout the test (Fig. 1). Neuronal firing was recorded on FM tape (Racal) and firing rates were plotted on-line by computer (IBM PS/2–70–121) together with markers for ionophoresis.

All the neurons recorded were located in dorsal horn laminae IV/V, had excitatory receptive fields on the ipsilateral hind limb (excluding glabrous skin)

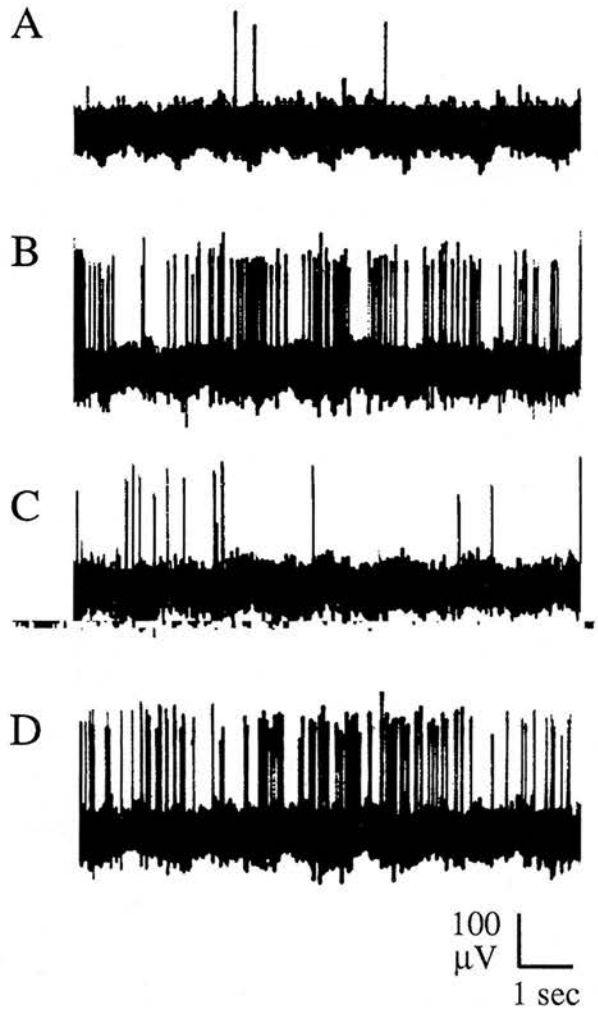


Fig. 1 Typical example of original oscilloscope records showing increased firing of the neuron in response to mustard oil and its inhibition by the NK-2 antagonist L 659,874. Trace A shows background spontaneous activity of this (multireceptive) neuron prior to testing. B shows activity 5 min after topical application of 7% mustard oil to the extent of the brush receptive field of the cell. In this example, mustard oil-evoked activity was sustained at this elevated level for a prolonged period without re-application. C shows activity 2 min after ionophoresis of L659,874 was initiated, 18 min following the application of mustard oil. D shows recovery, 10 min after L 659,874 ionophoresis was terminated at the end of test C.

and were multireceptive (i.e. displayed responses to both noxious and innocuous stimuli). Neurons were initially found by their responsiveness to widespread manual brushing of the ipsilateral hindlimb. Cutaneous fields of the neurons were first localised by responses to manual brush, then the more restricted pinch and noxious heat receptive fields were identified. Interestingly, six neurons which failed to show a response to noxious heat, gave no overt response to mustard oil, whereas in virtually all (> 95%) of other cases there was concurrent responsiveness to both of these stimuli. The mustard oil (5–20% in paraffin oil) was applied topically within the brush receptive field of neurons, with the intention of covering this firing field (usually about 3 cm^2). Thus, the mustard oil application encompassed the noxious heat/pinch receptive fields which were a sub-set of the brush field.

Experiments were routinely carried out with a range of ionophoretic currents of the antagonists, the aim being to increase currents stepwise until clear effects were seen. Drugs were usually ejected for 1–2 min periods in order to assess any maintained inhibition if present. When currents displaying a clear inhibition for at least 30 s were reached, ionophoresis was stopped to look for recovery. Ejection of apparently inactive compounds was carried out at ionophoretic currents equal to or greater than those for active inhibitor. In 17 cells, both NK_1 and NK_2 antagonists were tested, with the former being first in 12 cases and the latter being first in the remainder. The chosen order of application appeared to have no effect on the results. Mustard oil (3-isothiocyanato-prop-1-ene) was obtained from Aldrich Chemical Company.

Results

The present results were obtained from 35 neurons located in dorsal horn laminae IV/V. After application of mustard oil (5–20% in paraffin oil), virtually all the multireceptive neurons tested (34/35) had a large and prolonged increase in activity with firing rates of 138 ± 33-fold of background (mean ± SEM) being consistently maintained in the majority of cases (28 cells) for at least 28 min. Some cells had an immediate increase in activity after one application of mustard oil which remained sustained for more than 7 min (Figs 1, 2B & 3A). However, in most cases (n = 22), the steady elevated firing rate was achieved by repeated application of mustard oil 3–5 times to the same site over a period of 10–15 min. This apparently different sensitivity to mustard oil had no obvious effects on the pharmacological results obtained.

Three modified peptide antagonists highly selec-

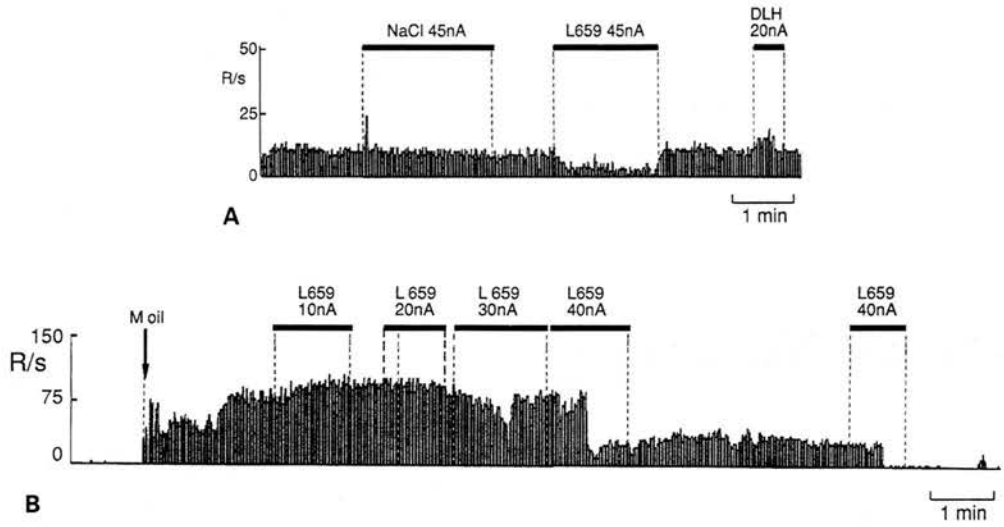


Fig. 2 Typical inhibitory effects of the NK-2 antagonist L 659,874 on mustard oil-evoked activity of laminae IV/V dorsal horn neurons. The records display raw firing frequency of the neuron (as spikes per sec; R/S) and its activation by either a single application (Fig. 1B) or 3 consecutive prior applications (Fig. 1A) of mustard oil. In (A) ionophoretic application of NaCl had no effect, whereas L 659,874 promptly caused greater than 50% inhibition of evoked activity. After terminating ionophoresis of L 659,874 rapid recovery was observed and the cell readily responded to a presumed direct excitatory stimulus d, I-homocysteic acid (DLH). In (B) mustard oil-evoked activity was progressively inhibited (and the inhibition maintained for longer periods) by ionophoresis of L 659,874 at increasing currents.

tive for NK_1 and NK_2 receptors were tested by ionophoresis close to the recorded cells. L 659,874 was described by McKnight et al[33] to show more than 200-fold and 250-fold selectivity for NK_2 over NK_1 and NK_3 sites respectively.[33] Its even more potent and selective cyclic congener L 659,877[34] was also inves-

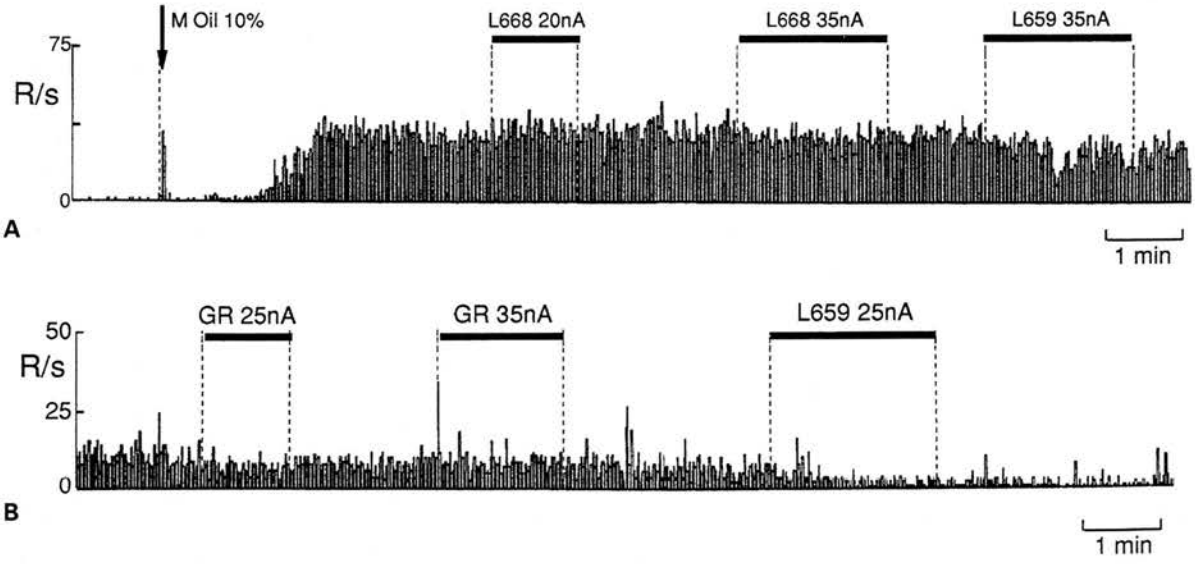


Fig. 3 Typical examples of the generally-observed lack of effect of NK_1 antagonists on the mustard oil-evoked activity of laminae IV/V dorsal horn neurons. The large increases in firing frequency elicited by mustard oil were unaffected by the NK_1 antagonists L 668,169 (A) and GR 82334 (B) applied after the mustard oil-induced activity had reached a steady state. In each case, the NK_2 antagonist L 659,874 caused inhibition. This effect was transient in example (A) and more long-lasting in (B), although in the latter example greater recovery was seen after a further delay of 3 min (not shown).

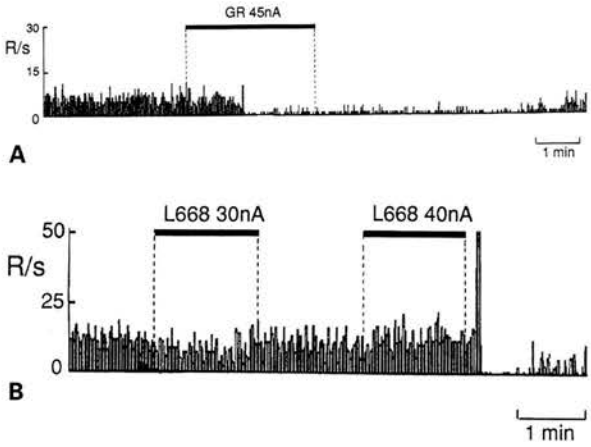


Fig. 4 Examples of the occasionally-observed inhibitory effect of NK_1 antagonists on mustard oil-evoked activity of laminae IV/V dorsal horn neurons. A clear-cut inhibition of mustard oil-evoked firing was seen in 3 out of 11 cases with GR 82334 as in example (A). Delayed recovery was observed. In only one example, (B), was there any evidence for inhibitory effects of L 668,169 and even there the inhibition was not closely matched to the time of drug application.

tigated but could not be tested due to its low solubility in acceptable vehicles. Drugs were tested after mustard oil-evoked activity was stable and had been continuous for at least 1–2 min. In 23 out of 24 cells tested with the NK_2 antagonist L 659,874 (10–80 nA) there was a clear and relatively maintained inhibition of mustard oil-evoked activity (Figs 1 & 2) to 50 ± 7% (mean ± SEM) of prior control activity (each averaged over three 10-second periods). Inhibition began after an onset delay of 0–120 s (mean = 38 ± 9 s, n = 23). In 19 cases where currents of ≥ 40 nA were tested, clear inhibition was always apparent within 30 s. Recovery was observed in 13 out of 17 cases examined after terminating ionophoresis of L 659,874 (Fig. 2A) and in about half of these cases (n = 6), inhibition was maintained at its maximal level for no more than 30–60 s (mean = 48 ± 6 s) even in the continued presence of L 659,874 (Figs 2B & 3A). Increasing the ejection currents however, produced a more marked inhibition in 15 out of 19 neurons tested, with a typical example showing 17, 33 and 85% inhibition at 20, 30 and 40 nA respectively (see also Fig. 2B). The inhibition was also longer lasting at higher ionophoretic currents and in all cells tested with ≥ 40 nA L 659,874 (n = 17) the effect was fully maintained throughout the ejection period. In 4 examples no recovery was observed, but throughout this period the cells were still able to generate vigorous responses to manual brush stimuli. Neither current controls (from the NaCl barrel) nor vehicle controls at equivalent ionophoretic currents had any discernible effect on mustard oil-evoked activity (n = 3 in each case).

In contrast, two NK_1 selective antagonists L 668,169 and GR 82334 (20–80 nA) rarely inhibited mustard oil-evoked activity (in 1 out of 11 and 3 out of 11 cases respectively). L 668,169 is reported to show 80-fold and 25-fold selectivity for NK_1 over NK_2 and NK_3 receptors respectively[33] and equivalent values for GR 82334 are greater than 170-fold in each case.[35] In 17 cells tested with both NK_1 and NK_2 antagonist, there was only one example where both were clearly effective. The mustard oil-evoked activity in many cells (n = 15) was unaffected by NK_1 antagonists but inhibited by the NK_2 antagonist L 659,874 (Fig. 3). In a small number of examples (n = 4) there was unequivocal inhibition of activity by NK_1 antagonists (Fig. 4), although in the one effective example with L 668,169, inhibition was not certainly related to the time of administration of the drug (Fig. 4B). In this example the cell was lost while waiting for recovery.

Discussion

These results suggest that NK_2, but not NK_1 receptors, play a crucial role in mediating the sustained excitation of lamina IV/V dorsal horn neurons elicited by cutaneous application of mustard oil. As configured in these experiments, the mustard oil stimulus represents a medium term prolonged activation of nociceptive afferents. Evidence has been presented that mustard oil elicits activation of largely C-afferents[32,36] although it is possible that some Aδ activation also occurs transiently.[37] It is of interest that stimulation of cutaneous nociceptors with mustard oil has been described to lead to central sensitisation of dorsal horn neurons and expansion of their effective receptive fields.[38] Those experiments were designed slightly differently, in that mustard oil was applied to regions close to but outwith the overt firing zones. However, the neurons that exhibited mustard oil-evoked plasticity of receptive fields or responsiveness were either caused to fire or displayed increased spontaneous subthreshold activity in response to mustard oil, sug-

gesting that just as here, a C-fibre mediated input was important in the responses being investigated. Both brush and pinch receptive fields and/or neuronal responsiveness were reported to be increased by mustard oil application.[38] Although dorsal horn NK_2 rather than NK_1 receptors seem important in mustard oil-evoked increases in spontaneous activity here, it is not clear whether NK_1 receptors may play a role in central sensitisation.

It is of course also possible that the localised route of administration of drugs here did not allow the NK_1 antagonists to access their (perhaps slightly more distant) sites of action. In tests of behavioural analgesia, certain peptide and non-peptide NK_1 antagonists elevate response thresholds.[23–26] SP is a potent excitant of laminae IV/V nociceptive neurons[27] and on intrathecal administration SP produces behaviour suggestive of noxious irritation.[28] Pinch rather than noxious heat stimuli release SP into dorsal horn[13,14] and SP-like immunoreactivity has been observed in identified A δ as well as C afferents;[29] consistent with the possibility that a somewhat different subset of nociceptive afferents is involved in pinch-evoked SP release from those in the heat- and mustard oil-evoked neuronal activity we have observed to be sensitive to NK_2 antagonists. Nevertheless, neither brief neuronal responses to pinch[17–20] nor nociceptive flexor reflexes[22] appear to be directly prevented by NK_1 antagonists. Instead the facilitation of nociceptive flexor reflexes elicited by conditioning C-fibre stimulation is inhibited by the antagonist spantide II[22] which shows a modest selectivity for NK_1 over NK_2 receptors.[39] This is consistent with evidence that SP (NK_1) antagonists prevent the secondary behavioural hyperalgesia following heat injury[30] and the late prolonged but not the brief epsps induced in dorsal horn neurons by C-fibre activation or noxious stimuli.[16,31]

In view of evidence for differences in the absolute potency of certain NK_1 antagonists between species, we tested GR 82334, which in contrast to L 668,169 (and indeed also spantide II) shows only slightly reduced potency in the rat bladder compared to guinea-pig and rabbit tissue bioassays.[40,41] Nevertheless, the present results with a medium-term prolonged chemical algogenic stimulus support previous observations on responses to brief noxious thermal stimuli[17–20] in their marked sensitivity to NK_2 rather than NK_1 receptor agents; thereby suggesting that somewhat different inputs or circumstances are involved here from those in experiments showing NK_1 sensitivity. Consistent with this theme, the facilitation of a nociceptive flexor reflex induced by intrathecal administration of NKA or by conditioning stimulation of gastrocnemius nerves at C-fibre intensity[42] and the excitation of dorsal horn neurons evoked by application of capsaicin to the dorsal root ganglia[43] are all inhibited by selective NK_2 receptor antagonists. The detailed characteristics and precise location of this NK_2-like receptor in dorsal horn remain to be ascertained.

Acknowledgements

This work was supported by the Wellcome Trust. We thank the Wellcome Animal Unit, R(D)SVS for facilities, Colin Warwick for preparation of diagrams and Marianne Eastwood for typing the manuscript. We are grateful to Les Iversen (Merck, Sharp and Dohme) and Phil Birch (Glaxo Group Research) for gifts of compounds.

References

1. Nawa, H., Kotani, H. and Nakanishi, S. Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing. Nature 1984; 312: 729–734.
2. Krause, J. E., Carter, M. S., Xu, Z. S. and MacDonald, M. R. Structure, expression and regulation of the rat preprotachykinin I gene. In: Henry, J. L., Couture, R., Cuello, A. C., et al. (eds) Substance P and Neurokinins. Springer, New York. p5–7.
3. Arai, H. and Emson, P. C. Regional distribution of neuropeptide K and other tachykinins (neurokinin A, neurokinin B and substance P) in rat central nervous system. Brain Research 1986; 399: 240–249.
4. Iversen, L. L. Multiple receptors for tachykinins. In: Hakanson, R. and Sundler, F. (eds) Tachykinin Antagonists. Elsevier, Amsterdam. 1985; 291–304.
5. Ogawa, T., Kanazawa, I. and Kimura, S. Regional distribution of substance P, neurokinin A and neurokinin B in rat spinal cord, nerve roots and dorsal root ganglia, and the effects of dorsal root or spinal transection. Brain Research 1985; 359: 152–157.
6. Kimura, A., Okada, M., Sugita, Y., Kanazawa, I. and Munekata, E. Novel neuropeptides, neurokinin α and β, isolated from porcine spinal cord. Proc Japan Acad 1983; 59(B): 101–104.
7. Sundler, F., Brodin, E., Ekblad, E., Hakanson, R. and Uddman, R. Sensory nerve fibres: distribution of substance P, neurokinin A and calcitonin gene-related peptide. In: R. Hakanson and F. Sundler (ed) Tachykinin Antagonists. Elsevier, Amsterdam. 1985; 3–14.
8. Duggan, A. W., Hope, P. J., Jarrott, B., Schaible, H. -G. and Fleetwood-Walker, S. M. Release, spread and persistence of immunoreactive neurokinin A in the dorsal horn of the cat following noxious cutaneous stimulation: studies with antibody microprobes. Neuroscience 1990; 35: 195.
9. Yashpal, K., Dam, T. -V. and Quirion, R. Quantitative autoradiographic distribution of multiple neurokinin binding sites in

rat spinal cord. Brain Res. 1990; 506: 259.
10. Poosch, M. S., Goebel, D. J. and Bannon, M. J. Distribution of neurokinin receptor gene expression in the rat brain. Society of Neuroscience Abstracts 1991; 17: 806.
11. Takeda, Y. and Krause, J. E. Pharmacological and molecular biological studies on the diversity of rat tachykinin NK-2 receptor subtypes in rat CNS, duodenum, vas deferens and urinary bladder. Ann. N Y Acad. Sci. 1991; 632: 479.
12. Tsuchida, K., Shigemoto, R., Yokata, Y. and Nakanishi, S. Tissue distribution and quantitation of the mRNAs for three rat tachykinin receptors. Eur. J. Biochem. 1990; 193: 751–757.
13. Kuraishi, Y., Hirota, N., Sato, Y., Hanashima, N., Takagi, H. and Satoh, M. Stimulus specificity of peripherally-evoked substance P release from the rabbit dorsal horn in situ. Neuroscience 1989; 30: 241.
14. Duggan, A. W., Hendry, I. A., Morton, C. R., Hutchison, W. D. and Zhao, Z. Q. Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. Brain Res. 1988; 451: 261.
15. Schaible, H. -G., Jarrott, B., Hope, P. J. and Duggan, A. W. Release of immunoreactive substance P in the spinal cord during development of acute arthritis in the knee joint of the cat; a study with antibody microprobes. Brain Res. 1990; 529: 214.
16. De Koninck, Y. and Henry, J. L. Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons in vivo by noxious stimulation. Proc. Nat. Acad. Sci. USA 1991;88: 11344.
17. Fleetwood-Walker, S. M., Mitchell, R., Hope, P. J., El-Yassir, N., Molony, V. and Bladon, C. M. The involvement of neurokinin receptor subtypes in somatosensory processing in the superficial dorsal horn of the cat. Brain Res. 1990; 519: 169–182.
18. Fleetwood-Walker, S. M., Mitchell, R., Hope, P. J., El-Yassir, N., Molony, V. and Bladon, C. M. The involvement of neurokinin receptor subtypes in somatosensory processing in the superficial dorsal horn of the cat: corrigendum. Brain Res. 1992; 579: 357.
19. Fleetwood-Walker, S. M., Hope, P. J., Parker, R. M. C. and Mitchell, R. Effects of antagonists selective for neurokinin-1 and neurokinin-2 receptors on somatosensory responses of rat dorsal horn neurons. Br. J. Pharmacol. 1991; 104: 472.
20. Fleetwood-Walker, S. M., Parker, R. M. C., Munro, F., Hope, P. J. and Mitchell, R. Evidence for a role of NK-2 receptors in mediating brief nociceptive inputs to rat dorsal horn (laminae III-V) neurons. Eur. J. Pharmacol. 1992 (Submitted).
21. Murase, K., Ryu, P. D. and Randic, M. Tachykinins modulate multiple ionic conductances in voltage-clamped rat dorsal horn neurons. J. Neurophys. 1989; 61: 854.
22. Wiesenfeld-Hallin, Z., Xu, X. -J., Hakanson, R., Feng, D. -M. and Folkers, K. The specific antagonistic effect of intrathecal spantide II on substance P- and C-fibre conditioning stimuli-induced facilitation of the nociceptive flexor reflex in rat. Brain Res. 1990; 526: 284.
23. Yamamoto, T. and Yaksh, T. L. Stereospecific effects of a non-peptide NK-1 selective antagonist CP-96,345: antinociception in the absence of motor dysfunction. Life Sci. 1991; 49: 1955.
24. Garret, C., Carruette, A., Fardin, V., Moussaoui, S., Peyronel, J. -F., Blanchard, J. -C. and Laduron, P. M. Pharmacological properties of a potent and selective nonpeptide substance P antagonist. Proc. Natl Acad. Sci. USA 1991; 88: 10208.
25. Murray, C. W., Cowan, A. and Larson, A. A. Neurokinin and NMDA antagonists (but not a kainic acid antagonist) are antinociceptive in the mouse formalin model. Pain 1991; 44: 179.
26. Post, J. and Folkers, K. Behavioural and antinociceptive effects of intrathecally injected substance P analogues in mice. Eur. J. Pharmacol. 1985; 113: 335–342.
27. Henry, J. L. Effects of substance P on functionally-identified units in cat spinal cord. Brain Res. 1976; 114: 439–451.
28. Hylden, J. L. K. and Wilcox, G. L. Intrathecal substance P elicits a caudally-directed biting and scratching behaviour. Brain Res. 1981; 217: 212–215.
29. Lawson, S. N., Waddell, P. J. and McCarthy, P. W. A comparison of the electrophysiological and immunocytochemical properties of rat dorsal root ganglion fibers with A and C fibers. In: R. F. Schmidt, H. -G. Schaible and C. Vahle-Hinz (ed) Fine Afferent Nerve Fibers and Pain. Weinheim. 1987: 193–203.
30. Coderre, T. J. and Melzack, R. Central neural mediators of secondary hyperalgesia following heat inury in rats: neuropeptides and excitatory amino acids. Neuroscience Letters 1991; 131: 71–74.
31. Urban, L. and Randic, M. Slow excitatory transmission in rat dorsal horn: possible mediation by peptides. Brain Res. 1984; 290: 336–341.
32. Heapy, C. G., Jamieson, A. and Russell, N. J. W. Afferent C-fibre and A-delta activity in models of inflammation. Br. J. Pharmacol. 1987; 90: 164P.
33. McKnight, A. T., Maguire, J. J., Williams, B. J., Foster, A. C., Tridgett, R. and Iversen, L. L. Pharmacological specificity of synthetic peptides as antagonists at tachykinin receptors. Regulatory Peptides 1988; 22: 127.
34. McKnight, A. T., Maguire, J. J., Elliott, N. J. et al. Pharmacological specificity of novel, synthetic, cyclic peptides as antagonists at tachykinin receptors. Br. J. Pharmacol. 1991; 104: 355–360.
35. Hagan, R. M., Ireland, S. J., Bailey, F., McBride, C., Jordon, C. C. and Ward, P. A spirolactam conformationally-constrained analogue of physalaemin which is a peptidase-resistant, selective neurokinin NK-1 receptor antagonist. Br. J. Pharmacol. 1991; 102: 168P.
36. Woolf, C. J. and Wall, P. D. The relative effectiveness of C primary afferent fibres of different origins in evoking a prolonged facilitation of the flexor reflex in the rat. J. Neurosci. 1986; 6: 1433–1443.
37. Harris, N. C. and Ryall, R. W. Mustard oil excites but does not inhibit nociceptive dorsal horn neurones in the rat: a presumed effect on A-delta fibres. Br. J. Pharmacol. 1988; 94: 180–184.
38. Woolf, C. J. and King, A. E. Dynamic alterations in the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat spinal cord. J. Neurosci. 1990; 10: 2717–2726.
39. Maggi, C. A., Patacchini, R., Feng, D. M. and Folkers, K. Activity of spantide I and II at various tachykinin receptors and NK-2 tachykinin receptor subtypes. Eur. J. Pharmacol. 1991; 199: 127–129.
40. Patacchini, R., Santicioti, P., Astolfi, M., Rovero, P., Viti, G. and Maggi, C. A. Activity of peptide and non-peptide antagonists at peripheral NK-1 receptors. Eur. J. Pharmacol. 1992; 215: 93–98.
41. Beresford, I. J. M., Ireland, S. J., Stables, J. M., Stubbs, C. M., Ball, D., Hagan, R. M. and Birch, P. J. Investigation into differences in tachykinin NK_1 receptors between and within species using a peptide and a non-peptide NK_1 receptor antagonist. Br. J. Pharmacol. 1992; 106: 92P.
42. Xu, H. -J., Maggi, C. A. and Wiesenfeld-Hallin, Z. On the role of NK-2 tachykinin receptors in the mediation of spinal reflex excitability in the rat. Neuroscience 1991; 44: 483.
43. Urban, L., Maggi, C. A., Nagy, I. and Dray, A. The selective NK-2 receptor antagonist MEN 10,376 inhibits synaptic excitation of dorsal horn neurons evoked by C-fibre activation in the in vitro spinal cord. Neuropeptides 1992; 22: 68.

P82 The Role of NK_1 and NK_2 Receptors in Nociceptive Inputs to Rat Dorsal Horn Lamina I and Laminae IV/V Neurons

S. Fleetwood-Walker, R. Parker, F. Munro, M. Young and R. Mitchell*
Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh and *MRC Brain Metabolism Unit, George Square, Edinburgh, UK

Single neurons of chloralose/urethane anaesthetised rats were extracellularly-recorded and drugs applied by ionophoresis.[1] In lamina I, [Met-OMe^{11}]SP (NK_1 agonist) had no effect in 9/10 cells whereas NKA and GR 64349 (NK_2) produced a marked but transient excitation in nearly all cases. However, in laminae IV/V, [Ac-Arg^6, Sar^9, $Met(O_2^{11})$]SP_{6-11}(NK_1), NKA and GR 64349 were all effective excitants. In experiments with antagonists on laminae IV/V neurons, L-659874 (NK_2) selectively inhibited brief thermal nociceptive responses in 9/9 cases but L-668169 (NK_1) had no effect in 7/9. Similar results were obtained in models of sustained neuronal activation by mustard oil[2] or induction of preprodynorphin mRNA by intraplantar carrageenan.[3] Our results suggest that NK_2 but not NK_1 receptors may be important in nociceptive responses of laminae I and IV/V neurons to brief thermal and modest inflammatory stimuli. NK_1 receptors may be more important in profound inflammatory conditions, since in contrast to NKA, SP is released only by damaging levels of thermal stimuli.

1. Fleetwood-Walker, S. M., Hope, P. J., Parker, R. M. C. and Mitchell, R. (1991). Effects of antagonists selective for NK_1 and NK_2 receptors on somatosensory responses of rat dorsal horn neurons. Br. J. Pharmacol. 104: 472P.
2. Munro, F. E., Fleetwood-Walker, S. M., Parker, R. M. C. and Mitchell, R. Mustard oil-evoked activation of rat dorsal horn neurons is inhibited by NK_2 but not NK_1 receptor antagonists. Br. J. Pharmacol (in press).
3. Parker, R. M. C., Fleetwood-Walker, S. M., Rosie, R., Munro, F. E. and Mitchell, R. Effects of NK receptor antagonists on carrageenan-induced expression of preprodynorphin mRNA in rat dorsal hom lamina I neurons. Br. J. Pharmacol, (in press).

299.6/O

EVIDENCE FOR THE INVOLVEMENT OF NK_2 RATHER THAN NK_1 RECEPTORS IN BOTH ACUTE AND SUSTAINED NOCICEPTIVE INPUTS TO DORSAL HORN NEURONS

FLEETWOOD-WALKER, S., PARKER, R., MUNRO, F., YOUNG, M. and MITCHELL, R.*

*Department of Preclinical Veterinary Sciences, Royal Dick School of Veterinary Sciences, University of Edinburgh, Edinburgh EH9 1QH, and *MRC Brain Metabolism Unit, University Department of Pharmacology, 1 George Square, Edinburgh EH8 9JZ, UK*

Both substance P (SP) and neurokinin A (NKA) are present in fine somatosensory afferents and can be released into spinal dorsal horn by noxious cutaneous stimuli. However, for thermal stimuli, detectable release of SP is not measured until skin temperatures reach levels likely to cause inflammatory damage. Here we assessed the role of NK_1/NK_2 receptors in acute and sustained (inflammatory) nociceptive inputs to dorsal horn neurons, using ionophoresis of NK receptor drugs. In acute experiments, the NK_2 antagonist L-659874 but not L-668169 (NK_1) inhibited thermal nociceptive responses of extracellularly-recorded cells without modifying responses to pinch or brush. Agonists for both NK_1 and NK_2 receptors activated laminae IV/V neurons however, suggesting that under some circumstances SP is likely to play some role in nociception. Interestingly, only NK_2 and not NK_1 agonists excited lamina I neurons. The sustained activity of laminae IV/V neurons 'wound-up' by repeated application of mustard oil to the periphery (over 10-30 min) was inhibited by L-659874 but not L-668169 or GR 82334 (NK_1). Neuronal activation elicited by intraplantar carrageenan (6 h) was assessed by in situ hybridisation for preprodynorphin (PPD) mRNA. The increased expression of PPD mRNA in superficial dorsal horn neurons was inhibited by L-659874 but not L-668169 or GR 82334. It is clear that NK_2 receptors are important in acute thermal nociception and in sustained inflammatory nociception of moderate intensity. It is not clear whether NK_1 receptors may be of greater importance in more profound inflammatory states.

RECEPTOR AND CELLULAR MECHANISMS INVOLVED IN MUSTARD OIL-INDUCED ACTIVATION OF DORSAL HORN NEURONS. F. Munro, M. Young, S. Fleetwood-Walker, R. Parker and R. Mitchell* Dept of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH and *MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ.

Aim of Investigation: The C-fibre-selective chemical irritant mustard oil produces sustained activity of dorsal horn neurons upon repeated peripheral application and long-lasting changes in receptive fields and responsiveness. We addressed the possible role of NK_1, NK_2 and metabotropic excitatory amino acid receptors and of protein kinase C (PKC) in responses to mustard oil.

Methods: Mustard oil was applied to restricted distal areas of one hindlimb of rats. In some experiments tissue was taken for biochemical assay of the translocation/activation of PKC assessed by movement of [^{3}H]phorbol dibutyrate ([^{3}H]PDBu) binding sites from cytosolic to membrane fractions. Extracellular recordings of single dorsal horn neurons were made in anaesthetised rats and drugs applied by ionophoresis.

Results: Selective antagonists for NK_2 and metabotropic receptors, L-659874 and l-AP3 reliably inhibited neuronal activity 'wound-up' by repeated application of mustard oil (in 19/21 and 6/9 cases respectively). The NK_1 antagonists, L-668169 and GR 82334 had no effect in 13/17 cases. The selective PKC inhibitors chelerythrine and GF 109203X caused marked, yet recoverable, inhibition of activity in 8/9 and 8/8 cases respectively. In corresponding spinal segments, significantly greater proportions of [^{3}H]PDBu binding sites were associated with the membrane fraction on the side ipsilateral to mustard oil application.

Conclusion: Both NK_2 and metabotropic receptors appear to play a role in the sustained activation of dorsal horn neurons elicited by mustard oil. There is good evidence that PKC is a crucial mediator of this cellular activation. NK_1 receptors appear to be of less importance in this model of sustained nociception.

EVIDENCE FOR A ROLE OF NK_2 RECEPTORS IN MEDIATING ACUTE AND SUSTAINED NOCICEPTIVE INPUTS TO DORSAL HORN NEURONS. S. Fleetwood-Walker, R. Parker, F. Munro, M. Young and R. Mitchell* (SPON: A. Iggo), Dept. of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH and *MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ.

Aim of Investigation: In view of reports that tachykinins with selectivity for NK_1 and NK_2 receptors (substance P and neurokinin A respectively) are contained in fine somatosensory afferents and can be released upon noxious stimulation, we assessed the role of these receptors in nociceptive processing.

Methods: The activation of single dorsal horn neurons during cutaneous stimulation was investigated in two models (i) acute natural stimuli to the peripheral receptive field (over 10-30 s) and (ii) peripheral inflammation induced by intraplantar injection of carrageenan (over 6 h). Experiments were carried out in anaesthetised rats. Neuronal responses were measured in model (i) by extracellular electrical recording and in model (ii) by in situ hybridisation histochemistry for preprodynorphin (PPD) mRNA. Antagonists selective for NK_1 or NK_2 receptors were delivered by ionophoresis into the spinal dorsal horn.

Results: Acute sensory responses to noxious heat but not innocuous brush or noxious pinch were inhibited by the NK_2 antagonist L-659874 (9/9 cells) but not by L-668169 (NK_1; no effect in 7/9 cells), confirming earlier work in cats. In 3 out of 3 animals with bilateral carrageenan inflammation, unilateral L-659874 inhibited the increased expression of PPD mRNA in ipsilateral superficial dorsal horn whereas L-668169 or GR 82334 were ineffective.

Conclusion: These results indicate that dorsal horn NK_2 receptors play a more important role than NK_1 in the present models of acute and sustained nociception. However, since laminae IV/V (although not lamina I) cells are excited by NK_1 as well as NK_2 agonists it seems likely that NK_1 receptors do play some role in nociceptive processing, perhaps under circumstances of more profound inflammation than assessed here.

REPRINTED FROM:

European Journal of Pharmacology

European Journal of Pharmacology, 242 (1993) 173–181

EJP 53306

Evidence for a role of tachykinin NK_2 receptors in mediating brief nociceptive inputs to rat dorsal horn (laminae III-V) neurons

Susan M. Fleetwood-Walker [a], Rachel M.C. Parker [a], Fiona E. Munro [a], Marie R. Young [a], Philippa J. Hope [a] and Rory Mitchell [b]

[a] *Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK and* [b] *MRC Brain Metabolism Unit, Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ, UK*

Received 29 April 1993, revised MS received 9 July 1993, accepted 16 July 1993

ELSEVIER SCIENCE PUBLISHERS B.V.— AMSTERDAM — LONDON — NEW YORK — TOKYO

European Journal of Pharmacology

The EUROPEAN JOURNAL OF PHARMACOLOGY publishes manuscripts on the mechanisms of action of chemical substances affec
biological systems.
Manuscripts submitted to the journal are only accepted on the understanding that: (1) they are subject to editorial review; (2) they have not b
and will not be published in whole or in part in any other journal; (3) the recommendations from the declaration of Helsinki and
internationally accepted principles in the care and use of experimental animals have been adhered to.

European Journal of Pharmacology, 242 (1993) 173–181

EJP 53306

Evidence for a role of tachykinin NK_2 receptors in mediating brief nociceptive inputs to rat dorsal horn (laminae III-V) neurons

Susan M. Fleetwood-Walker [a], Rachel M.C. Parker [a], Fiona E. Munro [a], Marie R. Young [a], Philippa J. Hope [a] and Rory Mitchell [b]

[a] *Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK and* [b] *MRC Brain Metabolism Unit, Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ, UK*

Received 29 April 1993, revised MS received 9 July 1993, accepted 16 July 1993

Since the NK_2 receptor-selective tachykinin, neurokinin A is present in fine primary afferent neurons in addition to the NK_1 receptor-selective tachykinin, substance P, we have addressed the relative role of NK_1 and NK_2 receptors in somatosensory processing in spinal dorsal horn. Recording extracellularly from rat laminae III–V neurons whilst ionophoresing drugs nearby, the selective NK_1 receptor antagonists L 688,169, GR 82334 and [D-Pro^4,D-Trp^{7,9,10}Phe^{11}]substance P-(4–11) failed to influence neuronal responses to cutaneous pinch or noxious heat but often enhanced responses to innocuous brush. In contrast, the highly selective NK_2 receptor antagonist L 659,874 profoundly inhibited responses to noxious heat but not pinch or brush. Highly selective synthetic agonists for both NK_1 and NK_2 receptors ([N-acetyl-Arg^6,Sar^9,Met(O$_2$)^{11}]substance P-(6–11) and GR 64349, respectively) and also NKA showed the inverse effects on sensory responses to those brought about by their antagonists. At higher ionophoretic currents, both NK_1 and NK_2 receptor agonists increased spontaneous activity. This increased basal firing induced by GR 64349 and neurokinin A (but not that due to [N-acetyl-Arg^6,Sar^9,Met(O$_2$)^{11}]substance P-(6–11) appeared to partially pre-empt further excitatory responses to noxious heat. It is concluded that although both NK_1 and NK_2 receptors can clearly mediate excitation of dorsal horn neurons, it is not NK_1, but rather NK_2 receptors that are important as the physiological transducer of brief thermal nociceptive inputs in this model.

Tachykinins; Neurokinins; Pain; Analgesia; Spinal cord

1. Introduction

The tachykinins substance P and neurokinin A (which display selectivity for NK_1 and NK_2 receptors respectively) are both present in fine somatosensory afferents (Hökfelt et al., 1975; Sundler et al., 1985) and for some time substance P has been a candidate transmitter of nociception (Henry, 1976). There is considerable evidence from electrophysiological, behavioural, biochemical and transmitter release experiments that substance P is involved in nociception (see Fleetwood-Walker and Mitchell, 1989, for review). Nevertheless, the crucial experiments with antagonists have provided mixed results. Early substance P antagonists [D-Pro^2,D-Trp^{7,9}]substance P and [D-Pro^2,D-Phe^7,D-Trp^9]substance P failed to reliably reverse the excitatory effects of substance P on trigeminal neurons (Hill et al., 1985) and [D-Arg^1,D-Trp^{7,9}Leu^{11}]substance P (spantide) failed to antagonise the substance P facilitation of the nociceptive flexor reflex in vivo (Wiesenfeld-Hallin and Duranti, 1987) and only inhibited nociceptive reflexes in vitro at rather high concentrations (Otsuka and Yanagisawa, 1988; Brugger et al., 1990). However, recent NK_1 receptor antagonists spantide II [D-NicLys^1,3-Pal^3,D-Cl$_2$Phe^5,Asn^6,D-Trp^{7,9},Nle^{11}]substance P (Maggi et al., 1991b) and CP 96,345 (Snider et al., 1991) have been described to block the facilitation of the flexor reflex and the excitation of dorsal horn neurons brought about by electrical stimulation of afferents at C-fibre intensity (Wiesenfeld-Hallin et al., 1990; De Koninck and Henry, 1991). In behavioural reflex experiments only certain of the early substance P antagonists effective elsewhere on smooth muscle preparations, elevated response thresholds to nociceptive stimuli (Post and Folkers, 1985; Piercey et al., 1986), although several newer NK_1 receptor antagonists were effective in models of inflammatory pain (Yamamoto and Yaksh, 1991; Murray et al., 1991;

Correspondence to: S.M. Fleetwood-Walker, Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK. Tel. 031 650 6091, fax 031 650 6576.

Garret et al., 1991; Birch et al., 1992; Boyce et al., 1992).

Little has been done to address any possible role of NK_2 receptors in dorsal horn, but ionophoretic administration of two early NK_2 receptor antagonists [D-Pro^4,Lys^6,D-Trp^{7,9,10},Phe^{11}]substance P-(4–11) and [D-Tyr^4,D-Trp^{7,9},Nle^{11}]substance P-(4–11) (Regoli et al., 1985) but not an NK_1 receptor antagonist, spantide, into the substantia gelatinosa, specifically inhibited the responses of cat dorsal horn neurons to noxious thermal, but not other, cutaneous stimuli (Fleetwood-Walker et al., 1990).

Whilst several reports have described direct excitatory effects of substance P on dorsal horn neurons (Henry, 1976; Murase et al., 1989; Urban and Dray, 1992), not only the selective NK_1 receptor agonist [Met-OMe^{11}]substance P (Fleetwood-Walker et al., 1990) but also the NK_2 receptor agonist neurokinin A (Salter and Henry, 1991) have been reported to excite dorsal horn lamina IV/V neurons when ionophoretically ejected nearby.

The present experiments were carried out with a range of recently developed agonists and antagonists which are highly selective for NK_1 and NK_2 receptors in order to further address their relative contribution to mediating spinal nociceptive transmission.

2. Materials and methods

2.1. General

Experiments were carried out, broadly as described previously (Fleetwood-Walker et al., 1991), on male rats (270–350 g), anaesthetised with intravenous α-chloralose (60 mg kg^{-1}) and urethane (1.2 mg kg^{-1}), after induction with halothane. Supplementary doses of α-chloralose were given as required. Core temperature was maintained at 37–38°C with a thermostatically controlled heated blanket, and in the majority of experiments carotid blood pressure was monitored throughout the experiment. Oxygen (0.1 l min^{-1}) was passed over the end of the tracheal cannula to enrich the inspired air. The thoraco-lumbar spinal column was supported by three pairs of clamps. A laminectomy (segments L1–L4) was then carried out and agar injected under the most rostral clamped vertebra and then over the whole area of the laminectomy. A core of agar was removed from above the recording region, the dura carefully cut and a pool of liquid paraffin applied to the region.

2.2. Electrophysiological methods and ionophoresis

Extracellular recordings were made via the central barrel (4 M NaCl, pH 4.0–4.5) of a 7-barrelled glass microelectrode. Electrode tip sizes were 4.0–4.5 μm and DC resistances were 5–8 MΩ. The band-width of the recording amplifier was 1 Hz–7 kHz. One side barrel contained 1 M NaCl (pH 4.0–4.5) for automatic current balancing and current controls (Neurophore Ionophoresis System, Medical Systems Corporation). Another side barrel contained Pontamine Sky Blue dye (2% in 0.5 M sodium acetate for marking recording sites by ejection for 100 μA min). The locations of recording sites in laminae III–V verified histologically in 15 μm cryostat sections lightly counterstained with neutral red. Other barrels contained the following neurokinin receptor agonists and antagonists: neurokinin A; L 659,874 (acetyl-Leu,Met,Gln,Trp,Phe-NH_2), L 668,169 (cyclo(Gln,D-Trp,Me-Phe,(R)Gly[ANC-2]Leu,Met)$_2$), L 659,877 (cyclo(Gln,Trp,Phe,Gly,Leu,Met)) (all from Cambridge Research Biochemicals); [D-Pro^4,D-Trp^{7,9,10},Phe^{11}]substance P-(4–11) (from Bachem UK); GR 82334 ([D-Pro^9[spiro-γ-lactam]-Leu^{10},Trp^{11}]physalaemin-(1–11) and GR 64349 ([Lys^3,Gly^8-R-γ-lactam-Leu^9]neurokinin-(3–10) (gifts from Glaxo Group Research) and [N-acetyl-Arg^6,Sar^9, Met(O$_2$)^{11}]substance P-(6–11) (a gift from Professor D. Regoli). Solutions were either 1 mM in distilled water (GR 82334, neurokinin A and GR 64349) or 0.1 mM in 0.3% dimethylformamide, diluted from concentrated stocks in pure dimethylformamide (other compounds) and were adjusted to pH 4.5 with HCl. All peptide solutions were either freshly made or stored at −20°C in small (100 μl) aliquots which once thawed were not refrozen, or used again. All drugs were applied with cathodal currents. Retaining currents of −10 nA were used to minimise drug leakage between tests. Action potentials of the recorded cells were clearly discriminated from other field potentials throughout the test. Neuronal firing was recorded on FM tape (Racal) and firing rates were plotted on-line by computer (IBM PS/2-70-121) together with stimulator and ionophoresis markers.

All the neurons recorded were selected as having cutaneous excitatory receptive fields on the hairy skin of the ipsilateral hind limb that were multireceptive (i.e. mediated responses to both noxious and innocuous stimuli). Receptive fields were initially located by brief manual brush/pinch stimuli, prior to characterisation with timed and quantified stimuli, including also noxious heat. Controlled stimuli were applied to adjacent cutaneous areas within the receptive field on the dorsal surface of the paw or the hind limb were regularly repeated over 3–4 min cycles. The innocuous stimulus used was a motorised, rotating brush. Noxious stimuli were provided by a calibrated pinch (serrated forceps with a graduated controlled displacement) or a thermocouple-controlled radiant heat lamp (giving a skin surface temperature ramp of 30–48°C). Like the other stimuli, the noxious heat stimulus was set routinely for

10 s at 48°C, but in occasional examples for 15 s to ensure a more robust response. Skin temperatures never exceeded the 45°C definition of noxious levels (Duggan et al., 1988) for longer than 1.5 s beyond the 10 or 15 s duration of the pre-set 48°C stimulus. The marker bars in the figures indicate the period of skin temperature in excess of 45°C. The cycle of stimuli was repeated every 3 min, with the ejection of the drugs initiated 1 min before the start of a cycle and continuing through that cycle. The different responses and epochs of spontaneous activity prior to each cycle of stimuli were integrated over 10–15 s periods as appropriate and compared to pre-drug control values, meaned from at least two consecutive tests. The stimulus-evoked responses were always submaximal and approximately matched in terms of neuronal firing rates. Duplicate or triplicate control responses were required to vary by less than 15–20%.

3. Results

The present results were obtained from a total of 61 neurons which were all located in laminae III–V of the dorsal horn. Peptide antagonists highly selective for NK_1 and NK_2 receptors (L 668,169/GR 82334/[D-Pro^4,D-Trp^{7,9,10}Phe$_{11}$]substance P-(4–11) and L 659,874, respectively; Regoli et al., 1987; McKnight et al., 1988; Hagan et al., 1991) were tested by ionophoresis close to the recorded cells. The vehicle (0.3% dimethylformamide in water) had no effect on 3 out of 3 neurons when ejected at up to 80 nA for 12 min. The rather more potent cyclic analogue of L 659,874, L 659,877, could not be tested because of its low solubility in acceptable vehicles.

Fig. 1 shows typical results (displayed as raw activity records) obtained with the NK_1 receptor antagonist L 668,169 in 6 out of 8 neurons tested. There was little effect of this antagonist on spontaneous activity or any sensory-evoked response apart from a small but consistent enhancement of activity evoked by innocuous brush (fig. 1, table 2). In the remaining 2 cells there were no detectable changes. In 4 cells where recovery was assessed there was 50% (n = 1) and over 85% (n = 3) reversal of the effect of L 668,169 on brush responses by 4 and 9–24 min respectively, after cessation of ionophoresis. Entirely similar results were obtained with GR 82334 in 5 out of 6 cells, showing a small increase in responses to brush, but no effect on those to noxious heat (fig. 2, Table 2). In 2 neurons tested with [D-Pro^4,D-Trp^{7,9,10},Phe$_{11}$]substance P-(4–11) (30–40 nA, 6 min) brush responses were increased by 33 and 41% with no consistent change in other activity. Ionophoretic currents up to 50, 45 and 80 nA were tested for L 668,169, GR 82334 and [D-Pro^4,D-Trp^{7,9,10},Phe^{11}]substance P-(4–11) respectively, until either spike reduction or technical factors prevented further study. The effects on sensory responses at these currents were similar to those at lower currents, notably there still being no consistent inhibition of reponses to noxious heat.

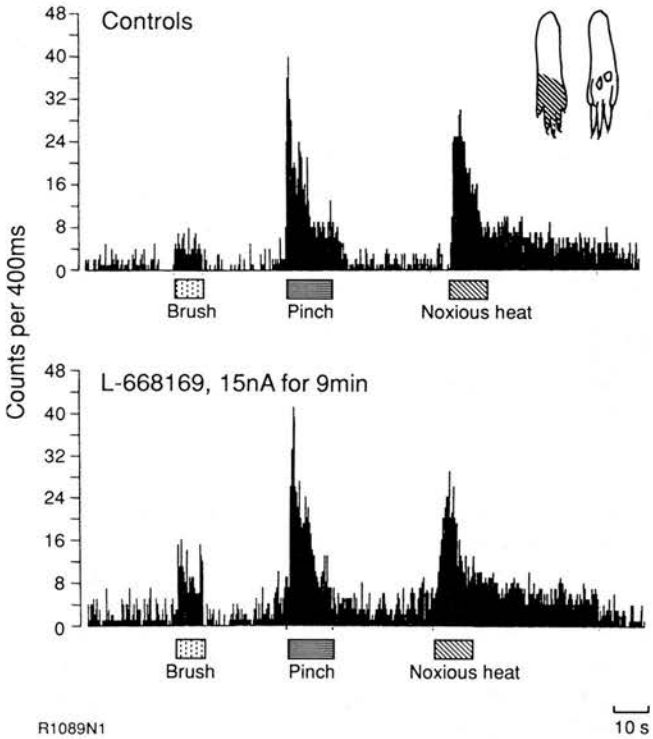


Fig. 1. Ongoing firing frequency records showing typical effects of the NK_1 receptor antagonist L 668,169 on somatosensory responses of a laminae III–V neuron. A modest facilitation of responses to innocuous brush was observed in 6 out of 8 neurons tested, with no other consistent changes. Recovery from drug effects was not examined closely, but in 3 out of 4 cells examined, responses to brush had recovered to within ±15% of control levels within 9–24 min after stopping ionophoresis of the drug. The fourth cell showed approximately 50% recovery within 4 min. The inset shows the position of the cutaneous receptive field on the ipsilateral hind limb. The duration of each of the stimuli is indicated, in the case of noxious heat, this representing the period for which the skin temperature exceeded the generally accepted noxious threshold of 45°C.

The NK_2 receptor antagonist L 659,874 produced a quite different result: a highly selective inhibition of responses to noxious thermal stimuli without altering responses to noxious pinch, innocuous brush or spontaneous activity (fig. 3, table 2). This was seen in 8 out of 10 neurons tested at just 10 nA of L 659,874 (mean 49% inhibition), with no detectable effect in the others. At a higher ejection current (20 nA) the selective inhibition was seen in all cells tested (9 out of 9) and was of a greater degree (mean 66% inhibition) (this was particularly clear in 4 cases where both currents were tested on the same cell). In 3 neurons where recovery was investigated, the inhibition of heat responses by L 659,874 had partially recovered (to 50–85% of controls) within 6–15 min after cessation of

TABLE 1

Time dependence of the effects of neurokinin receptor antagonists on thermal nociceptive responses.

All values represent evoked activity (corrected for background) and are shown as percentages of the mean stimulus-induced activity occurring during the stimulus. Values are expressed as mean ± S.E.M.

	Noxious heat-induced increase in neuronal firing rate (% of control response during stimulus)		
	During stimulus	0–10 s after stimulus	10–20 s after stimulus
(a) L 668,169 (n = 6)			
Control	100 (11 ± 1 s)	21 ± 7	15 ± 9
Drug (20 nA for 6 min)	90 ± 6	20 ± 11	9 ± 7
(b) GR 82334 (n = 5)			
Control	100 (10 s)	28 ± 7	9 ± 5
Drug (32 ± 4 nA for 6 min)	93 ± 10	23 ± 12	8 ± 7
(c) L 659,874 (n = 9)			
Control	100 (11 ± 1 s)	25 ± 9	11 ± 9
Drug (20 nA for 6 min)	36 ± 6	10 ± 6	7 ± 7

ionophoresis. In the majority of cells tested here, responses to noxious heat declined rapidly on cessation of the stimulus (to less than 20% of the response during the stimulus, within the subsequent 10 s). For each of the drugs tested there were, however, examples of relatively prolonged after discharges, retaining around 50% of the peak activity during the first post-stimulus period. There was no evidence that effects of any of the drugs were greater (or in any way different) in post-stimulus periods than during application of the heat stimulus (table 1).

A number of NK_1 and NK_2 receptor agonists were also examined (table 2). The highly selective NK_1 receptor agonist [N-acetyl-Arg^6,Sar^9,Met(O_2)^{11}]substance P-(6–11) (Regoli et al., 1988) was tested either on neuronal responses to cycled cutaneous stimuli either at low or higher ionophoretic currents. In 9 out of 13 cells [N-acetyl-Arg^6,Sar^9,Met(O_2)^{11}]substance P-(6–11) (when ejected at relatively low currents: mean 31 ± 4 nA) caused an inhibition of the activity evoked by innocuous brush with no apparent effect on spontaneous activity or nociceptive responses. In 6 out of 6 cells, higher ejection currents (mean 57 ± 5 nA) of [N-acetyl-Arg^6,Sar^9,Met(O_2)^{11}]substance P-(6–11) increased spontaneous activity, causing marked and sustained rises to around 5-fold of pre-drug controls. Recovery was observed in all 5 cases examined within 5–10 min after terminating ionophoresis and the drug effect was not replicated in current (NaCl) or vehicle control tests.

TABLE 2

Summary of the effects of neurokinin receptor antagonists and agonists on laminae III–V neurons.

All values are calculated as a percentage of the mean pre-drug control value and are expressed as the mean ± S.E.M. Responses were designated as being essentially unchanged if they remained within 20% of the mean control value. Neurons in the population tested which were not influenced in the characteristic predominant fashion illustrated, all showed no (or no consistent) changes in their responses. For the subpopulations of neurons affected by the drugs, the statistical significance of changes was assessed by Wilcoxon test.

Drug and conditions		Stimulus-evoked neuronal firing rate (% of mean pre-drug control)					
		Spontaneous	Brush	Pinch	Noxious heat	Number of neurons influenced	Number of neurons tested
Antagonists							
L 668,169 (NK_1)	20 nA for 6 min	95 ± 7	141 ± 9 [a]	105 ± 10	82 ± 9	6	8
GR 82334 (NK_1)	32 ± 4 nA for 6 min	103 ± 8	131 ± 8 [a]	–	91 ± 11	5	6
L 659,874 (NK_2)	10 nA for 6 min	93 ± 7	103 ± 9	99 ± 8	51 ± 6 [a]	8	10
	20 nA for 6 min	95 ± 12	104 ± 6	85 ± 11	34 ± 5 [a]	9	9
Agonists							
[Ac-Arg^6,Sar^9, Met(O_2)^{11}]-substance P-(6–11) (NK_1)	31 ± 4 nA for 5 ± 1 min	121 ± 13	52 ± 5 [a]	98 ± 5	92 ± 6	9	13
	57 ± 5 nA for 6 ± 2 min	536 ± 88 [a]	67 ± 9 [a]	91 ± 14	106 ± 11	6	6
Neurokinin A (NK_2)	11 ± 5 nA for 7 ± 1 min	112 ± 10	89 ± 11	108 ± 10	155 ± 13 [a]	6	6
	44 ± 4 nA for 6 ± 1 min	354 ± 61 [a]	79 ± 19	120 ± 21	64 ± 16 [a]	6	8
GR 64349 (NK_2)	66 ± 6 nA for 7 ± 1 min	398 ± 54 [a]	111 ± 13	88 ± 20	75 ± 11 [a]	5	5

[a] $P < 0.05$, compared to mean pre-drug control.

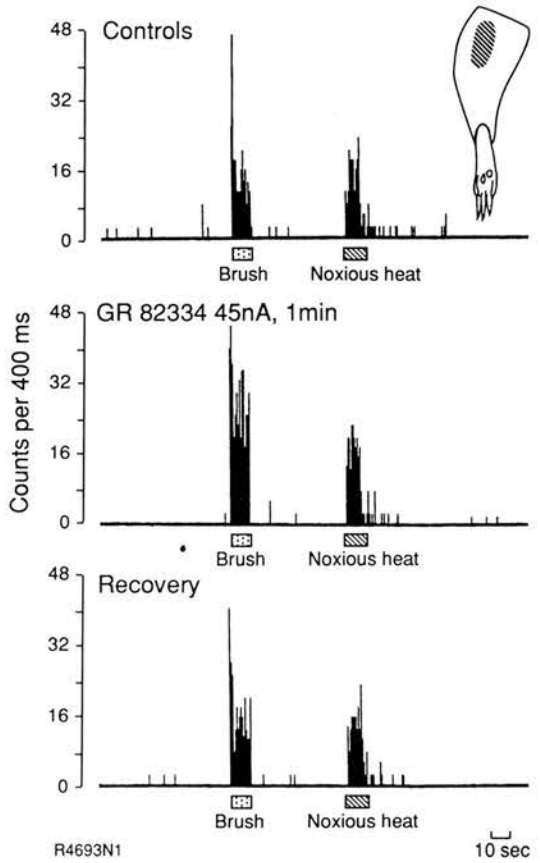


Fig. 2. Ongoing firing frequency records showing typical effects of the NK_1 receptor antagonist GR 82334 on somatosensory responses of a laminae III–V neuron. Facilitation of responses to innocuous brush (with no other consistent change) was observed in 5 out of 6 cells examined. The lower trace shows recovery 3 min after termination of ionophoresis. Similar effects were observed in 3 further cells where recovery was assessed. The inset shows the position of the cutaneous receptive field on the ipsilateral hind limb.

The NK_2 receptor agonists, neurokinin A and the novel highly selective compound GR 64349 (Hagan et al., 1991) also increased spontaneous activity of laminae III–V neurons in a consistent manner. Neurokinin A (44 ± 4 nA) and GR 64349 (66–6 nA) produced marked and sustained excitation of spontaneous activity in 6 out of 8 and 5 out of 5 cases respectively. When neurokinin A was tested at low currents (mean 11 ± 5 nA) which did not raise spontaneous activity in the recorded neuron, there was a selective facilitation of the responses to noxious thermal stimuli (in 6 out of 8 cells). Responses to innocuous brush and noxious pinch were essentially unaltered. Although one cell showed a 30% increase in the noxious pinch response, this was not reproduced in the other examples. At the higher ionophoretic currents of neurokinin A and GR 64349 that increased spontaneous activity, noxious heat-evoked responses were attenuated when corrected for the elevated baseline. Other responses were essentially unaltered. In 2 cases with neurokinin A, the increment in spontaneous activity was equivalent to the magnitude of reduction in heat responses. In all other cases this magnitude of reduction was greater (mean 3–9-fold) than the actual increase in spontaneous activity. Recovery was observed within 6–12 min after terminating ionophoresis and none of the effects was reproduced in current control tests. In 4 cells where [N-acetyl-Arg^6,Sar^9,Met(O$_2$)^{11}]substance P-(6–11) and neurokinin A were each tested, clear excitatory responses to both the NK_1 and NK_2 receptor agonist were seen.

Fig. 4 (representative of 3 out of 3 neurons) shows the selective facilitation of thermal nociceptive responses by a low current of neurokinin A and a reduction in those facilitated responses when L 659,874 was additionally applied. This is consistent with reversal of the effect of exogenous neurokinin A by the selective NK_2 receptor antagonist but cannot be unequivocal, since the antagonist alone had inhibitory effects on thermal nociceptive responses (fig. 3). Analogous results were obtained in 3 experiments when the effect of GR 82334 was tested on the inhibition of innocuous

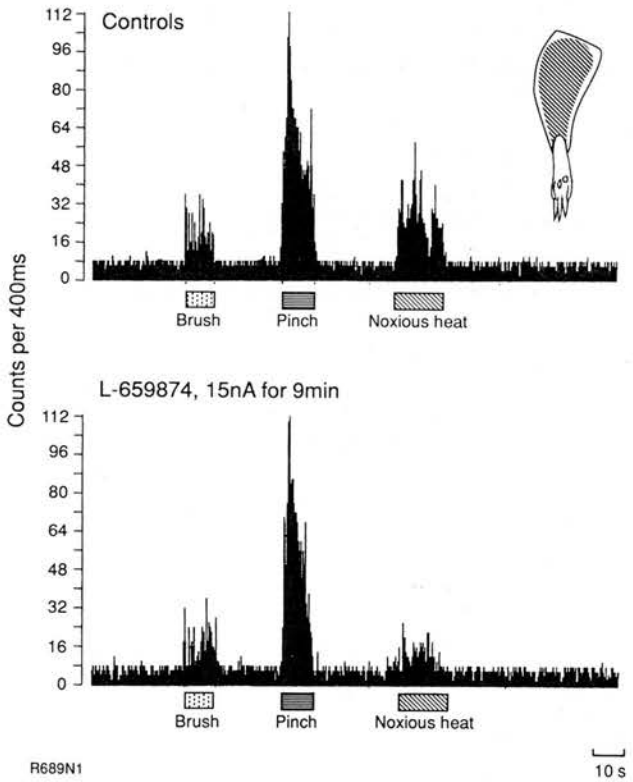


Fig. 3. Ongoing firing frequency records showing typical effects of the NK_2 receptor antagonist L 659,874 on somatosensory responses of a laminae III–V neuron. A marked attentuation of responses to noxious heat but not other stimuli or spontaneous activity was seen in all 11 neurons tested at currents of 15–20 nA. Recovery from drug effects was not examined closely, but in 3 out of 3 cells examined there was recovery of the noxious heat response to within 50–85% of control at 6–15 min after terminating ionophoresis of the drug. The inset shows the position of the cutaneous receptive field on the ipsilateral hind limb.

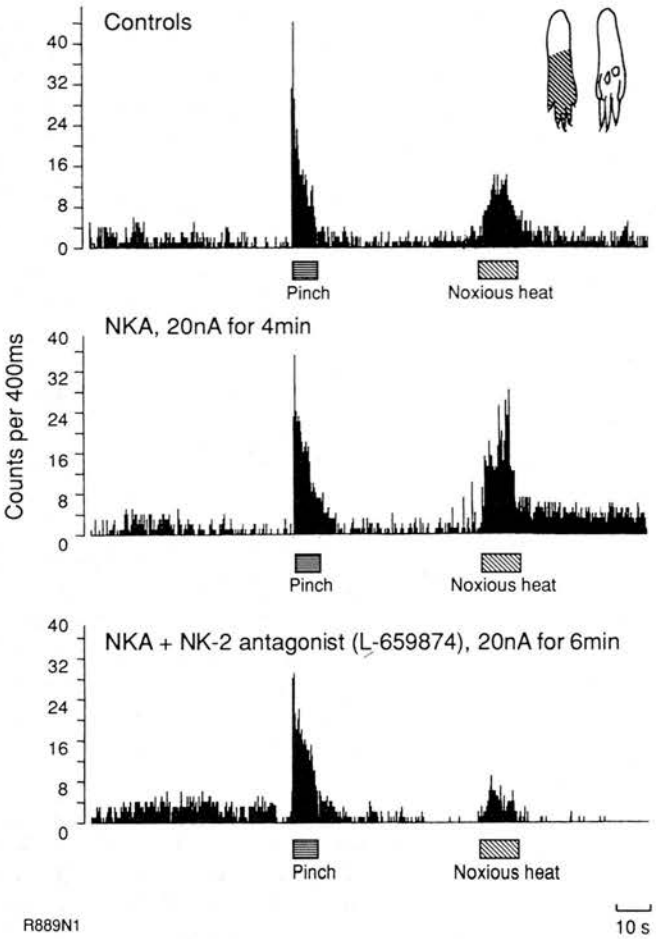


Fig. 4. Ongoing firing frequency records showing typical effects of the NK_2 receptor agonist neurokinin A, in the absence or presence of the NK_2 receptor antagonist L 659,874 on the somatosensory responses of a laminae III–V neuron. Neurokinin A caused a selective facilitation of responses to thermal but not mechanical noxious stimuli. Responses to innocuous brush (not shown here) were also unaltered (see table 2). Results were typical of 6 neurons tested with low currents of neurokinin A. After the middle trace had been recorded, ionophoresis of L 659,874 was begun in addition to neurokinin A. A marked attenuation of the neurokinin A-amplified response to noxious heat to below control levels was observed in all 3 cells so tested. The inset shows the position of the cutaneous receptive field on the ipsilateral hind limb.

brush responses induced by low currents of [N-acetyl-Arg^6,Sar^9,Met(O_2)^{11}]substance P-(6–11). Effects of antagonists on the increases in spontaneous activity brought about by high ejection currents of NK_1 and NK_2 receptor agonists were not investigated in the present study.

4. Discussion

These experiments provided no clear evidence that NK_1 receptors participate in transducing brief nociceptive inputs to laminae III–V neurons of rat spinal dorsal horn under the present conditions. Since the potency of some NK_1 receptor antagonists, including L 668,169, is reduced in rodent bioassays compared to those in other species (Pattachini et al., 1992), we also tested GR 82334 (which retains high potency at rat NK_1 receptors; Beresford et al., 1992) and [D-Pro^4,D-Trp^{7,9,10},Phe^{11}]substance P-(4–11) (which is effectively antinociceptive in the mouse formalin model; Murray et al., 1991). The NK_1 receptor antagonists L 668,169, GR 82334 and [D-Pro^4,D-Trp^{7,9,10}Phe^{11}]substance P-(4–11) had no significant effect on nociceptive responses in any neurons tested, but in contrast caused a modest facilitation of their responses to innocuous brush in the majority of cells. This suggests that their lack of effect on nociceptive responses was not due to failure to reach relevant sites at effective concentrations. Conviction that this effect of L 668,169, GR 82334, and [D-Pro^4,D-Trp^{7,9,10},Phe^{11}]substance P-(4–11) was due to NK_1 receptor antagonism is strengthened by that being the common property of structurally quite distinct compounds, by the low ionophoretic currents required, by the observation that NK_1 receptor agonists produce precisely the inverse effect (table 2) and by the apparent reduction in the effect of [N-acetyl-Arg^6,Sar^6,Met(O_2)^{11}] substance P-(6–11) in the presence of GR 82334.

The simplest interpretation of the facilitation of brush responses by NK_1 receptor antagonists seems to be that NK_1 receptors are responsible for an attenuating influence on non-nociceptive inputs to these cells and that the antagonist is acting to reverse the action of the endogenous NK_1 receptor agonist, substance P, which has been released during the course of our experimental procedure. The factors leading to substance P release here are unclear (see below), but substance P does not appear to be responsible for acutely mediating the increased activity elicited by brief thermal or mechanical noxious stimuli. It is of course possible that the antagonists have unknown side effects or that more complex interactions are occurring. The NK_1 receptor agonist [N-acetyl-Arg^6,Sar^9,Met(O_2)^{11}] substance P-(6–11) clearly increased the spontaneous activity of laminae III–V neurons here (table 2), indicating that NK_1 receptors can exert a direct (or at least a general modality-independent) influence on activity of these cells. The data indicating that NK_1 receptor activation can excite these cells, but seems not to participate in nociceptive responses and instead attenuates non-nociceptive tactile responses (in at least a subpopulation of cells) are entirely consistent, in our hands, between rat and cat. Somewhat in contrast to the present results, Radhakrishnan and Henry (1991) reported inhibition by CP-96,345 of the after-discharge following brief noxious mechanical or thermal stimuli. There was little effect on the initial phase of excitatory responses to brief heat stimuli and inconsistent effects on pinch-evoked excitation. It is possible that CP-96,345

is effective only on late components of nociceptive responses or that actions of this compound other than on NK_1 receptors may be involved. Indeed, there is new evidence that CP-96,345 is a potent antagonist of 'L'-type Ca^{2+} channels (Schmidt et al., 1992) and furthermore that such compounds are effective antinociceptive agents (Miranda et al., 1992). Although both peptide and non-peptide NK_1 receptor antagonists have been reported to block the late prolonged but not the brief excitatory postsynaptic potentials induced in dorsal horn neurons by C-fibre activation or by noxious stimuli (Urban and Randic, 1984; De Koninck and Henry, 1991), in our experiments, there was no evidence that NK_1 receptor antagonists were any more effective in the 20 s of afterdischarge following a noxious heat stimulus than they were during the response itself (table 1).

In experiments designed to examine adequate stimuli for the release of substance P into spinal cord, it has been reported that noxious pinch, intraplantar formalin, topical methylene chloride and flexion of a kaolin/carrageenan-inflamed knee-joint were effective (Duggan et al., 1988; Kuraishi et al., 1989; Schaible et al., 1990). Thermal cutaneous stimuli, however, were only effective at skin temperatures considered to result in inflammatory cutaneous lesions (Duggan et al., 1988; Kuraishi et al., 1989). In contrast, both noxious mechanical stimuli and noxious thermal stimuli (at skin temperatures below those producing inflammatory damage) were effective in releasing neurokinin A (Duggan et al., 1990). Behavioural reports have described inhibition by CP-96,345 of the second (inflammation-supported) phase of the response to intraplantar formalin (Yamamoto and Yaksh, 1991) and inhibition by [Arg^1,D-Pro^2,D-Phe^7,D-His^9]substance P of the secondary contralateral hyperalgesia following heat injury (Coderre and Melzack, 1991). Correspondingly, only the facilitation of nociceptive flexor reflex by substance P or C-afferent conditioning stimuli (and not the reflex itself) was inhibited by the NK_1 receptor antagonist spantide II (Wiesenfeld-Hallin et al., 1990). Thus, our evidence and the data from behavioural and release experiments point towards the involvement of substance P in mediating inflammation-supported nociception rather than responses to brief noxious stimuli.

In contrast to the results concerning NK_1 receptors, the NK_2 receptor antagonist L 659,874 caused a marked and highly consistent inhibition of nociceptive responses without affecting spontaneous activity or responses to innocuous brush. Inhibition by the NK_2 receptor antagonist showed a striking selectively for thermal rather than mechanical nociceptive stimuli, a result also noted in cat with the moderately selective NK_2 receptor antagonist [D-Pro^4,Lys^6,D-Trp^{7,9,10}, Phe^{11}]substance P-(4–11) (Fleetwood-Walker et al., 1990). The mechanistic basis for this is unclear, since polymodal C-afferents are likely to contribute greatly to both responses. Nevertheless, small dorsal root ganglion cells are very heterogeneous in terms of their neuropeptide content and there is evidence that thermal/mechanical noxious stimuli can differentially elicit release of neuropeptides into spinal perfusates (Kuraishi et al., 1989). Consistent with a role of an NK_2 receptor agonist such as neurokinin A in thermal, but not mechanical nociception is its selective facilitation of thermal nociceptive responses (fig. 4, a result also seen in cat with neurokinin A and other agonists with NK_2 receptor selectively (Fleetwood-Walker et al., 1990). In the current experiments, both neurokinin A and the highly selective NK_2 receptor agonist GR 64349 further caused marked and consistent increases in spontaneous activity of laminae III–V neurons, consistent with the idea that NK_2 receptor activation is an important element in the transduction of thermal nociceptive inputs. It is of course possible that co-factors may also subserve an important role. Interestingly, although both NK_1 and NK_2 receptor agonists increase the spontaneous activity in laminae III–V cells, the situation is quite different in lamina I where only NK_2 but not NK_1 receptor agonists are effective (Fleetwood-Walker et al., 1992).

There is increasing biochemical evidence that NK_2 receptors (although generally at much lower abundance than NK_1 or NK_3 receptor sites) are present in the CNS and in particular in the dorsal horn of the spinal cord (Yashpal et al., 1990; Poosch et al., 1991; Takeda and Krause, 1991). Additionally functional studies from other groups are now beginning to support a role for NK_2 receptors in spinal nociception. Xu et al. (1991) described that the NK_2 receptor antagonist, [Tyr^5,D-Trp^{6,8,9},Arg^{10}]neurokinin A-(4–10) (MEN 10207) selectively reversed the facilitation by neurokinin A (but not substance P) of the spinal nociceptive flexor reflex. Furthermore, a congener with further reduced partial efficacy [Tyr^5,D-Trp^{6,8,9},Lys^{10}]neurokinin A-(4–10) (MEN 10376; Maggi et al., 1991a) inhibited the synaptic excitation of dorsal horn neurons evoked by capsaicin administration to the dorsal root ganglia (but not that evoked by an NK_1 receptor agonist) in a spinal cord slice with attached dorsal roots (Urban et al., 1992).

The pharmacological properties of the small, but functionally important population of NK_2 receptors in dorsal horn remain to be investigated in detail.

Acknowledgements

This work was funded by the Wellcome Trust and the MRC. We also thank the Wellcome Animal Unit for facilities, Lucy Mitchell for technical help, Colin Warwick for preparation of diagrams and Anne Stirling-Whyte and Elaine Chalmers for typing the manuscript. We

are grateful to Les Iversen (Merck, Sharp & Dohme), Phil Birch (Glaxo Group Research) and Domenico Regoli (University of Sherbrooke) for gifts of compounds and to Mayenk Dutia (University of Edinburgh) for providing his neuronal analysis program.

References

Beresford, I.J.M., S.J. Ireland, J.M. Stables, C.M. Stubbs, D. Ball, R.M. Hagan and P.J. Birch, 1992, Investigation into differences in tachykinin NK_1 receptors between and within species using a peptide and a non-peptide NK_1 receptor antagonist, Br. J. Pharmacol. 106, 92P.

Birch, P.J., S.M. Harrison, A.G. Hayes, H. Rogers and M.B. Tyers, 1992, The non-peptide NK_1 receptor antagonist (±) CP-96,345 produces antinociceptive and anti-oedema effects in the rat, Br. J. Pharmacol. 105, 508.

Boyce, S., M.J. Rupniak, L. Young, W. Rycroft, L. Bristow, S.D. Iversen and R.G. Hill, 1992, Antinociceptive effects of racemic RP 67580, but not CP-96,345, on acetic acid-induced writhing and formalin paw in mice, Neuropeptides 22, 10.

Brugger, F., F.H. Evans and N.S. Hawkins, 1990, Effects of N-methyl-D-aspartate antagonists and Spantide on spinal reflexes and responses to substance P and capsaicin in isolated spinal cord preparations from mouse and rat, Neuroscience 36, 611.

Coderre, T.J. and R. Melzack, 1991, Central neural mediators of secondary hyperalgesia following heat injury in rats – neuropeptides and amino acids, Neurosci. Lett. 131, 71.

De Koninck, Y. and J.L. Henry, 1991, Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons in vivo by noxious stimulation, Proc. Natl. Acad. Sci. USA 88, 11344.

Duggan, A.W., I.A. Henry, C.R. Morton, W.D. Hutchison and Z.Q. Zhao, 1988, Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat, Brain Res. 451, 261.

Duggan, A.W., P.J. Hope, B. Jarrott, H.-G. Schaible and S.M. Fleetwood-Walker, 1990, Release, spread and persistence of immunoreactive neurokinin A in the dorsal horn of the cat following noxious cutaneous stimulation: studies with antibody microprobes, Neuroscience 35, 195.

Fleetwood-Walker, S.M. and R. Mitchell, 1989, Role of substance P in nociception, Current Opinion Anaesthesiol. 2, 645.

Fleetwood-Walker, S.M., R. Mitchell, P.J. Hope, N. El-Yassir, V. Molony and C.M. Bladon, 1990, The involvement of neurokinin receptor subtypes in somatosensory processing in the superficial dorsal horn of the cat, Brain Res. 519, 169; 579, 357.

Fleetwood-Walker, S.M., P.J. Hope, R.M.C. Parker and R. Mitchell, 1991, Effects of antagonists selective for neurokinin-1 and neurokinin-2 receptors on somatosensory responses of rat dorsal horn neurons, Br. J. Pharmacol. 104, 472P.

Fleetwood-Walker, S.M., R.M.C. Parker, F. Munro, R. Mitchell and P. Birch, 1992, Excitation of rat dorsal horn lamina I neurons by selective agonists at NK_2 but not NK_1 receptors, Neuropeptides 22, 23.

Garret, C., A. Carruette, V. Fardin, S. Moussaoui, J.F. Peyronel, J.-C. Blanchard and P.M. Laduron, 1991, Pharmacological properties of a potent and selective nonpeptide substance P antagonist, Proc. Natl. Acad. Sci. USA 88, 10208.

Hagan, R.M., S.J. Ireland, C.C. Jordan, I.J.M. Beresford, M.J. Deal and P. Ward, 1991, Receptor-selective, peptidase-resistant agonists at neurokinin NK_1 and NK_2 receptors: new tools for investigating neurokinin function, Neuropeptides 19, 127.

Henry, J.L., 1976, Effects of substance P on functionally-identified units in cat spinal cord, Brain Res. 114, 439.

Hill, R.G., T.E. Salt and G.E. Leighton, 1985, Actions of putative substance P antagonists on neurones in the CNS, in: Tachykinin Antagonists, eds. R. Hakanson and F. Sundler (Elsevier, Amsterdam) p. 377.

Hökfelt, T., J.O. Kellerth, G. Nilsson and B. Pernow, 1975, Substance P: localisation in the central nervous system and in some primary sensory neurons, Science 190, 889.

Kuraishi, Y., N. Hirota, Y. Sato, N. Hanashima, H. Takagi and M. Satoh, 1989, Stimulus specificity of peripherally-evoked substance P release from the rabbit dorsal horn in situ, Neuroscience 30, 241.

Maggi, C.A., S. Giuliani, L. Ballati, A. Lecci, S. Manzini, R. Patacchini, A.R. Renzetti, P. Rovero, L. Quartara and A. Giachetti, 1991a, In vivo evidence for tachykinergic transmission using a new NK_2 receptor-selective antagonist, MEN 10,376, J. Pharmacol. Exp. Ther. 257, 1172.

Maggi, C.A., R. Patacchini, D.-M. Feng and K. Folkers, 1991b, Activity of Spantide I and II at various tachykinin receptors and NK_2 tachykinin subtypes, Eur. J. Pharmacol. 199, 127.

McKnight, A.T., J.J. Maguire, B.J. Williams, A.C. Foster, R. Tridgett and L.L. Iversen, 1988, Pharmacological specificity of synthetic peptides as antagonists at tachykinin receptors, Regul. Pept. 22, 127.

Miranda, H.F., D. Bustamante, V. Kramer, T. Pelissier, H. Saavedra, C. Paeile, E. Fernandez and G. Pinardi, 1992, Antinociceptive effects of Ca^{2+} channel blockers, Eur. J. Pharmacol. 217, 137.

Murase, K., P.D. Ryu and M. Randic, 1989, Tachykinins modulate multiple ionic conductances in voltage-clamped rat dorsal horn neurons, J. Neurophysiol. 61, 854.

Murray, C.W., A. Cowan and A.A. Larson, 1991, Neurokinin and NMDA antagonists (but not a kainic acid antagonist) are antinociceptive in the mouse formalin model, Pain 44, 179.

Otsuka, M. and M. Yanagisawa, 1988, Effect of tachykinin antagonist on a nociceptive reflex in the isolated spinal cord-tail preparation of the newborn rat, J. Physiol. (London) 395, 255.

Patacchini, R., P. Santicioti, M. Astolfi, P. Rovero, G. Viti and C.A. Maggi, 1992, Activity of peptide and non-peptide antagonists at peripheral NK_1 receptors, Eur. J. Pharmacol. 215, 93.

Piercey, M.F., M.W. Moon, J.R. Blinn and P.J.K. Dobry-Shreur, 1986, Analgesic activities of spinal cord substance P antagonists implicate substance P as a neurotransmitter of pain sensation, Brain. Res. 385, 74.

Poosch, M.S., D.J. Goebel and M.J. Bannon, 1991, Distribution of neurokinin receptor gene expression in the rat brain, Soc. Neurosci. Abstr. 17, 806.

Post, C. and K. Folkers, 1985, Behavioural and antinociceptive effects of intrathecally injected substance P analogues in mice, Eur. J. Pharmacol. 113, 335.

Radhakrishnan, V. and J.L. Henry, 1991, Novel substance P antagonist, CP-96,345, blocks responses of cat spinal dorsal horn neurons to noxious cutaneous stimulation and to substance P, Neurosci. Lett. 132, 39.

Regoli, D., P. D'Orleans-Juste, G. Drapeau, S. Dion and E. Escher, 1985, Pharmacological characterisation of substance P antagonists, in: Tachykinin Antagonists, eds. R. Hakanson and F. Sundler (Elsevier, Amsterdam) p. 277.

Regoli, D., G. Drapeau, S. Dion and P. D'Orleans-Juste, 1987, Pharmacological receptors for substance P and neurokinins, Life Sci. 40, 109.

Regoli, D., P. D'Orleans-Just, S. Dion, N.-E. Rhaleb, N. Rouissi and G. Drapeau, 1988, Neurokinin selective agonists: discovery and pharmacologic applications, Regul. Pept. 22, 153.

Salter, M.W. and J.L. Henry, 1991, Responses of functionally identified neurones in the dorsal horn of the cat spinal cord to substance P, neurokinin A and physalaemin, Neuroscience 43, 601.

Schaible, H.-G., B. Jarrott, P.J. Hope and A.W. Duggan, 1990, Release of immunoreactive substance P in the spinal cord during

development of acute arthritis in the knee joint of the cat; a study with antibody microprobes, Brain Res. 529, 214.

Schmidt, A.W., S. McLean and J. Heym, 1992, The substance P receptor antagonist CP-96,345 interacts with Ca^{2+} channels, Eur. J. Pharmacol. 215, 351.

Snider, R.M., J.W. Constantine, J.A. Low, III, K.P. Longo, W.S. Lebel, H.A. Woody, S.E. Drozda, M.C. Desai, F.J. Vinick, R.W. Spencer and H.-J. Hess, 1991, A potent, nonpeptide antagonist of the substance P (NK_1) receptor, Science 251, 435.

Sundler, F., E. Brodin, E. Ekblad, R. Hakanson and R. Uddman, 1985, Sensory nerve fibres: distribution of substance P, neurokinin A and calcitonin gene-related peptide, in: Tachykinin Antagonists, eds. R. Hakanson and F. Sundler (Elsevier, Amsterdam) p. 3.

Takeda, Y. and J.E. Krause, 1991, Pharmacological and molecular biological studies on the diversity of rat tachykinin NK_2 receptor subtypes in rat CNS, duodenum, vas deferens and urinary bladder, Ann. NY Acad. Sci. 632, 479.

Urban, L. and A. Dray, 1992, Synaptic activation of dorsal horn neurons by selective C-fibre excitation with capsaicin in the mouse spinal cord in vitro, Neuroscience 47, 693.

Urban, L. and M. Randic, 1984, Slow excitatory transmission in rat dorsal horn: possible mediation by peptides, Brain Res. 290, 336.

Urban, L., C.A. Maggi, I. Nagy and A. Dray, 1992, The selective NK_2 receptor antagonist MEN 10,376 inhibits synaptic excitation of dorsal horn neurons evoked by C-fibre activation in the in vitro spinal cord, Neuropeptides 22, 68.

Wiesenfeld-Hallin, Z. and R. Duranti, 1987, [D-Arg^1,D-$Trp^{7,9}$, Leu^{11}]Substance P (Spantide) does not antagonize substance P-induced hyperexcitability of the nociceptive flexion withdrawal reflex in the rat, Acta Physiol. Scand. 129, 55.

Wiesenfeld-Hallin, Z., X.-J. Xu, R. Hakanson, D.-M. Feng and K. Folkers, 1990, The specific antagonistic effect of intrathecal spantide II on substance P- and C-fibre conditioning stimuli-induced facilitation of the nociceptive flexor reflex in rat, Brain Res. 526, 284.

Xu, H.-J., C.A. Maggi and Z. Wiesenfeld-Hallin, 1991, On the role of NK_2 tachykinin receptors in the mediation of spinal reflex excitability in the rat, Neuroscience 44, 483.

Yamamoto, T. and T.L. Yaksh, 1991, Stereospecific effects of a nonpeptide NK_1 selective antagonist CP-96,345: antinociception in the absence of motor dysfunction, Life Sci. 49, 1955.

Yashpal, K., T.-V. Dam and R. Quirion, 1990, Quantitative autoradiographic distribution of multiple neurokinin binding sites in rat spinal cord, Brain Res. 506, 259.

Reprinted from

ELSEVIER

Neuroscience Letters 170 (1994) 199–202

NEUROSCI. LETTERS

Evidence for a role of protein kinase C in the sustained activation of dorsal horn neurons evoked by cutaneous mustard oil application

Fiona E. Munro[a], Susan M. Fleetwood-Walker[a,*], Rory Mitchell[b]

[a]*Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK*

[b]*MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ, UK*

ELSEVIER SCIENTIFIC PUBLISHERS IRELAND LTD.

ELSEVIER

Neuroscience Letters 170 (1994) 199–202

NEUROSCIENCE LETTERS

Evidence for a role of protein kinase C in the sustained activation of rat dorsal horn neurons evoked by cutaneous mustard oil application

Fiona E. Munro[a], Susan M. Fleetwood-Walker[a,*], Rory Mitchell[b]

[a]*Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK*

[b]*MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ, UK*

Received 29 October 1993; Revised version received 26 January 1994; Accepted 7 February 1994

Abstract

The intracellular mechanisms involved in the sensitisation of spinal dorsal horn neurons brought about by sustained or repeated nociceptive inputs are unknown. The present experiments addressed any role of protein kinase (PKC) in sustained nociceptive responses of rat dorsal horn neurons by: (i) ionophoretic administration of PKC inhibitors whilst recording activity evoked by repeated cutaneous application of mustard oil; and (ii) assessing subcellular translocation of PKC evoked in spinal cord by cutaneous application of mustard oil. Both marked attenuation of mustard oil-induced neuronal activity by PKC inhibitors and selective translocation of PKC in spinal cord tissue ipsilateral to mustard oil application strongly supported a critical role of PKC in sustained nociceptive responses to mustard oil.

Key words: Protein kinase C; Phorbol ester; Spinal cord; Dorsal horn; Nociception; Hyperalgesia

Sustained or repetitive activation of nociceptive afferents brings about a state of hyperalgesia which is partly due to a sensitisation of central neurons in the dorsal horn of the spinal cord [20–22]. Peripheral administration of the C fibre-selective chemical algogen, mustard oil (3-isothiocyanato-prop-1-ene) [8,22] results in dorsal horn neurons displaying receptive field expansion and increased responsiveness to fixed stimuli [21]. We have shown previously that repeated cutaneous application of mustard oil (7–20%; 2–5 × 5 min intervals to the same site) leads to an intense activation of dorsal horn neurons which is then largely maintained over many tens of minutes [13]. Both neurokinin-2 (NK_2) and metabotropic glutamate (mGlu) receptors appear to participate in mediating such sustained mustard oil-evoked activity [13,23].

The increased responsiveness and prolonged after-discharges induced by repetitive C afferent stimulation ('wind-up') or by intraplantar formalin can be reversed by *N*-methyl-D-aspartate (NMDA) receptor antagonists [4,7]. The intracellular mechanisms by which neurokinins and excitatory amino acids might bring about sensitisation of dorsal horn neurons are not clear. Since both NK_2 receptors and some subtypes of mGlu receptors appear to act largely through phosphoinositide hydrolysis [2,16], the present experiments were carried out to assess whether protein kinase C (PKC) plays a significant role in sustained neuronal activation by mustard oil.

Extracellular recordings were made from histologically-identified laminae IV/V neurons in segments L1–L4 of male Wistar rats (260–320 g), anaesthetised with intravenous α-chloralose (60 mg/kg) and urethane (1.2 g/kg) as described previously [5,13]. Electrode side barrels contained selective PKC inhibitors (Novabiochem, Nottingham, Notts, UK): chelerythrine [9] (1 mM, pH 4.5–5.0) and GF 109203X [18] (0.2 mM in 0.2% dimethylformamide (DMF), pH 4.5–5.0) for ionophoresis. Other barrels contained 1 M NaCl for automatic current balancing or current controls, 0.2% DMF for vehicle controls and 2% Pontamine sky blue in 0.5 M sodium acetate for marking recording sites. All of the neurons tested were

*Corresponding author.

SSDI 0304-3940(94)00128-W

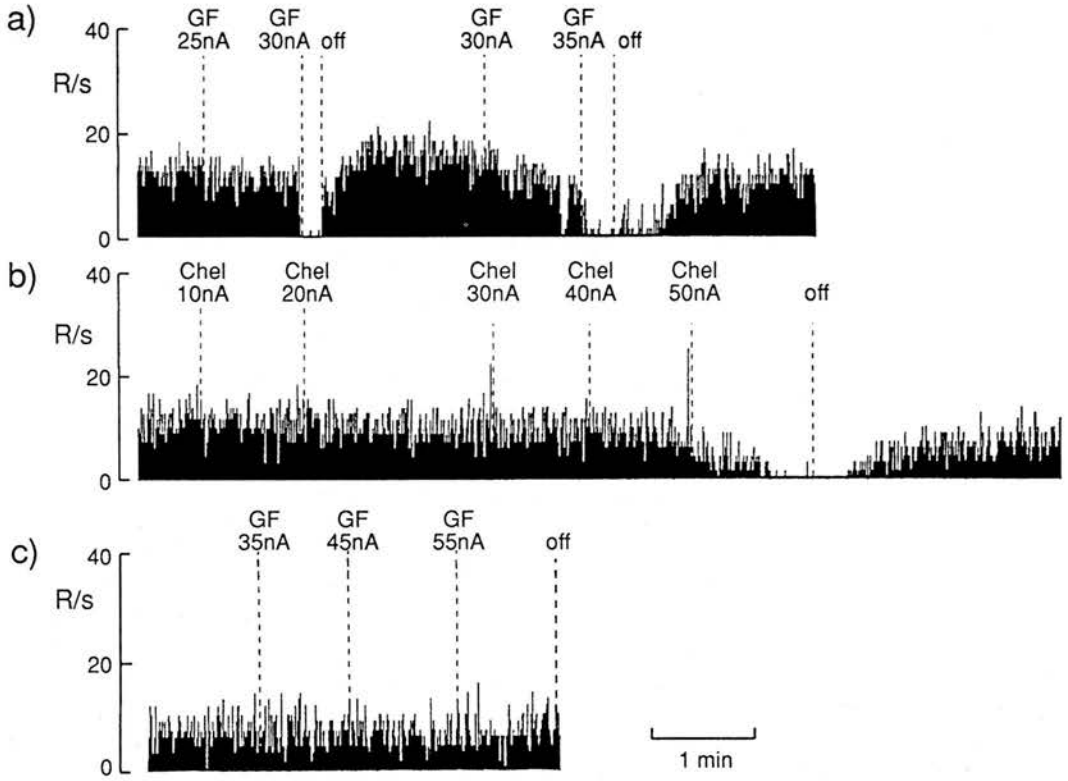


Fig. 1. Typical effects of protein kinase C (PKC) inhibitors, chelerythrine and GF 109203X on stimulus-evoked activation of multireceptive dorsal horn neurons. Continuous records of firing frequency (responses/s; R/S, bin width 1000 ms) are plotted against time. Prior to testing, all neurons examined showed a low spontaneous firing rate (<0.1 Hz). Activity was evoked in (a) and (b) by mustard oil (8.5%) applied to the receptive field 4 × 5 min intervals until a steady elevated firing rate was maintained or in (c) by motorised brush applied continuously to an equivalent receptive field area without mustard oil treatment. Drugs were applied ionophoretically at the indicated currents (maintained until the subsequent marker) and at all other times were subject to a retention current of −10 nA. Results are typical of 8 out of 8 cells and 4 out of 5 cells tested with GF 109203X (GF) in (a) and (c) respectively, and of 8 out of 9 cells tested with chelerythrine (Chel) in (b).

multireceptive (responding to brush, pinch and noxious 48°C heat) and had excitatory receptive fields on the hairy skin of the distal ipsilateral hindlimb. After characterising the field manually, mustard oil (Aldrich Chemical Co., Gillingham, Dorset) as a 7.5–10% solution in paraffin oil was applied (2–5 × 5 min intervals) to a restricted area (usually about 3 cm^2) within this region which had been shaved. In some experiments, responses to innocuous stimulation by continuous brushing were also examined.

Biochemical experiments were carried out to examine the effect of mustard oil on the subcellular translocation of [20–^{3}H(N)]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding sites from cytosolic to membrane compartments, which can be taken as an index of PKC activation in response to stimulation of cell surface receptors [11]. Male Wistar rats were anaesthetised with halothane and mustard oil (8% in paraffin oil) was applied 3 × 5 min intervals to an extensive area of shaved skin distal from the thigh on one hindlimb. The affected area was covered and after a further 30 min, the animal was killed by cervical fracture and spinal segments L2–L5 were carefully removed and hemisected on the midline for analysis. Tissue was homogenised and centrifuged before specific binding of [^{3}H]PDBu was determined as described previously [11].

PKC antagonists profoundly inhibited the mustard oil-evoked activity of dorsal horn neurons; generally within 1 minute of ionophoresis (Fig. 1). The inhibitory effects were defined at the 10 s period of greatest inhibition and were expressed as a percentage of control mustard oil-evoked activity in the 10 s period prior to drug administration. In 8 out of 9 cells tested with GF 109203X (20–60 nA), marked inhibition by 58 ± 9% (mean ± S.E.M.; range 34–99%) was seen. In 8 out of 8 cells tested with chelerythrine (10–80 nA) mustard oil-evoked activity was similarly inhibited by 70 ± 10% (mean ± S.E.M.; range 42–100%). In 4 out of 5 cells where GF 109203X was tested on brush-evoked activity, there was no discernible effect, with only a small inhibition (<15%) in the fifth cell. Chelerythrine was not examined on brush-evoked activity. In all cases recovery occurred rapidly within 1 min of removing the inhibitor, presumably indicating a requirement for ongoing PKC

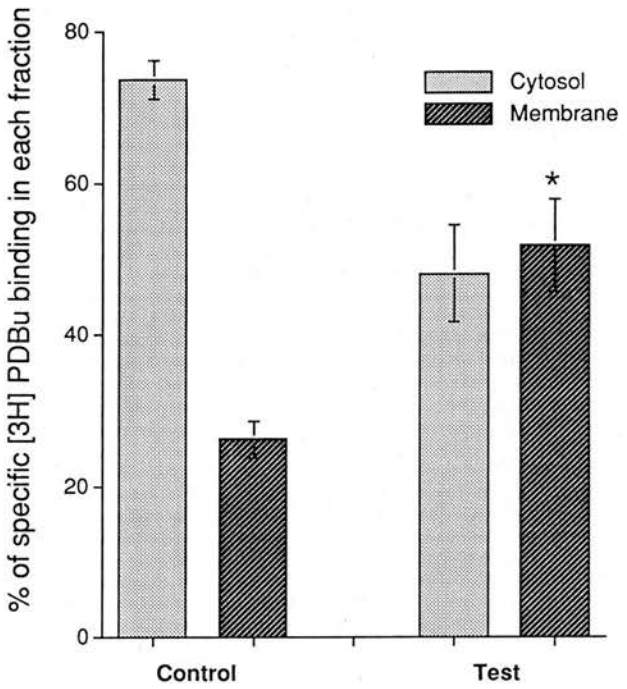


Fig. 2. The effect of unilateral mustard oil application to the skin of a distal hindlimb on the subcellular distribution of [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding sites in spinal cord. Specific binding of [^{3}H]PDBu was determined in homogenates of hemisected spinal cord (segments L2–L5). On the control side of spinal cord (contralateral to mustard oil) this represented 12,000–15,000 dpm/assay (>85% specific) in the cytosolic fraction and 4000–5000 dpm/assay (>75% specific) in the membrane fraction. On the test side of spinal cord (ipsilateral to mustard oil application), the absolute specific binding in the membrane fraction was increased on average by 55 ± 15% (mean ± S.E.M., $n = 9$) and that in the cytosolic fraction fell by 50 ± 13%, such that the total recovered specific binding was only slightly reduced (< 10%) from that on the control side. The proportion of specific binding recovered in the membrane fraction on the test side was significantly greater than the corresponding value on the control side ($P < 0.05$, Mann-Whitney U-test).

activity in mediating the mustard oil response rather than a role for long-lasting phosphorylation events. In 3 out of 3 cells tested, ionophoresis of vehicle or NaCl at up to 80 nA had no detectable effect on neuronal responses to cutaneous stimuli.

In the biochemical experiments, tissue was successfully obtained from 9 preparations and demonstrated a marked translocation of [^{3}H]PDBu binding sites from cytosolic to membrane fractions on the side ipsilateral to mustard oil compared to contralateral control (Fig. 2).

The present results show that cutaneous stimulation with mustard oil leads to translocation/activation of PKC in ipsilateral spinal cord and that the corresponding activation of ipsilateral dorsal horn neurons is consistently reversed by selective PKC inhibitors. Other groups have provided evidence consistent with a role of PKC in central sensitisation. In dorsal horn slices in vitro, phorbol esters facilitated depolarising responses to NMDA (in the presence of tetrodotoxin) and also increased the release of excitatory (and other) amino acids evoked by dorsal root stimulation [6]. The potentiation of depolarising NMDA responses produced by neurokinins in isolated dorsal horn neurons is mimicked by the PKC activator PDBu (but also by activators of other kinases) and inhibited by the non-selective kinase inhibitor staurosporine [15]. The nociceptive behaviour in rats following intraplantar injection of formalin was promoted by intrathecal administration of a phorbol ester and reduced by a non-selective kinase inhibitor H7 [3]. In contrast, in neonatal rat spinal cord in vitro the facilitation by neurokinin A of ventral root potentials evoked by bath-applied NMDA was unaffected by staurosporine [19]. Long-term increases in the levels of [^{3}H]PDBu binding sites have also been reported ipsilateral (and to a lesser extent also contralateral) to experimental constrictive neuropathy and to monoarthritis induced by Complete Freund's Adjuvant; consistent with an involvement of PKC in long-term central changes elicited by inflammation [12,17]. Interestingly the increased expression of preprodynorphin mRNA in ipsilateral superficial dorsal horn elicited by intraplantar carrageenan (like mustard oil-induced activation here [13]), is blocked by NK_2 receptor antagonists [14], again consistent with a role of a signal resulting from phosphoinositide hydrolysis such as PKC activation.

In conclusion, under the present circumstances at least, PKC is strongly implicated as a crucial mediator of the activation of dorsal horn neurons evoked by a sustained nociceptive challenge. It is not yet clear whether PKC activation results predominantly from neurokinin, excitatory amino acid or other receptor mechanisms. Although PKC activation is reported to diminish voltage-sensitive Mg^{2+} block of the NMDA channel [1] and may in some situations facilitate excitatory amino acid release [6,10], the PKC-dependent mechanisms underlying sustained activation here are unknown.

This work was supported by the Wellcome Trust. We thank the Wellcome Animal Research Unit, R(D)SVS for facilities, Colin Warwick for preparation of figures and Anne Stirling-Whyte for typing the manuscript.

[1] Ben-Ari, Y., Aniksztejn, L. and Bregestovski, P., Protein kinase C modulation of NMDA currents: an important link for LTP induction, Trends Neurosci., 15 (1992) 333–339.
[2] Bristow, D.R., Curtis, N.R, Suman-Chauhan, N., Watling, K.J. and Williams, B.J., Effects of tachykinins on inositol phospholipid hydrolysis in slices of hamster urinary bladder, Br. J. Pharmacol., 90 (1987) 211–217.
[3] Coderre, T.J., Contribution of protein kinase C to central sensitization and persistent pain following tissue injury, Neurosci. Lett., 140 (1992) 181–184.
[4] Davies, S.N. and Lodge, D., Evidence for involvement of *N*-methyl-aspartate receptors in 'wind-up' of class 2 neurones in the dorsal horn of the rat, Brain Res., 424 (1987) 402–406.
[5] Fleetwood-Walker, S.M., Parker, R.M.C., Munro, F.E., Young,

M.R., Hope, P.J. and Mitchell, R., Evidence for a role of NK_2 receptors in mediating brief nociceptive inputs to rat dorsal horn (laminae III–V) neurons, Eur. J. Pharmacol., 242 (1993) 173–181.

[6] Gerber, G., Kangrga, I., Ryu, P.D., Larew, J.S.A. and Randic, M., Multiple effects of phorbol esters in the rat spinal dorsal horn, J. Neurosci., 9 (1989), 3606–3617.

[7] Haley, J.E., Sullivan, A.F. and Dickenson, A.H., Evidence of spinal *N*-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat, Brain Res., 518 (1990) 218–226.

[8] Heapy, C.G., Jamieson, A. and Russell, N.J.W., Afferent C-fibre and A-delta activity in models of inflammation, Br. J. Pharmacol., 90 (1987) 164P.

[9] Herbert, J.N., Augereau, J.M., Gleye, J. and Maffrand, J.P., Chelerythrine is a potent and specific inhibitor of protein kinase C, Biochem. Biophys. Res. Commun., 172 (1990) 993–999.

[10] Herrero, I., Miras-Portugal, M.T. and Sanchez-Prieto, J., Positive feeedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation, Nature, 360 (1992) 163–166.

[11] Lutz, E.M., Mitchell, R., Johnson, M.S. and MacEwan, D., Functional expression of 5-HT_{1c} receptor cDNA in COS-7 cells and its influence on protein kinase C, FEBS Lett., 316 (1993) 228–232.

[12] Mao, J., Price, D.D., Mayer, D.J. and Hayes, R.L., Pain-related increases in spinal cord membrane-bound protein kinase C following peripheral nerve injury, Brain Res., 588 (1992) 144–149.

[13] Munro, F.E., Fleetwood-Walker, S.M., Parker, R.M.C. and Mitchell, R., The effects of neurokinin receptor antagonists on mustard oil-evoked activation of rat dorsal horn neurons, Neuropeptides, 25 (1993) 299–305.

[14] Parker, R.M.C., Fleetwood-Walker, S.M., Rosie, R., Munro, F.E. and Mitchell, R., Inhibition by NK_2 but not NK_1 antagonists of carrageenan-induced preprodynorphin mRNA expression in rat dorsal horn lamina I neurons, Neuropeptides, 25 (1993) 213–222.

[15] Rusin, K.I., Ryu, P.D. and Randic, M., Modulation of excitatory amino acid responses in rat dorsal horn neurons by tachykinins, J. Neurophysiol., 68 (1992) 265–286.

[16] Schoepp, D.D. and Conn, P.J., Metabotropic glutamate receptors in brain function and pathology. Trends Pharmacol. Sci., 14 (1993) 13–20.

[17] Tölle, T.R., Ableitner, A., Castro-Lopes, J.M., Coimbra, A. and Zieglgänsberger, W., Protein kinase C, c-Fos and prodynorphin mRNA are altered with distinct spatial and temporal patterns in the spinal cord of monoarthritic rats, Proceedings of the VIIth World Congress on Pain, Pain, August 1993, p. 475.

[18] Toullec, D., Pianetti, P., Coste, H., Bellevergue, P. Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D. and Kirilovsky, J., The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C, J. Biol. Chem., 266 (1990) 15771–15781.

[19] Urban, L, Naeem, S. and Dray, A., Effects of neurokinins on excitatory amino acid-induced activation in the neonatal rat spinal cord in vitro, Br. J. Pharmacol., 108 (1993) 23P.

[20] Woolf, C.J., Evidence for a central component of post-injury pain hypersensitivity, Nature, 308 (1983) 686–688.

[21] Woolf, C.J. and King, A.E., Dynamic alterations in the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat spinal cord, J. Neurosci., 10 (1990) 2717–2726.

[22] Woolf, C.J. and Wall, P.D., Relative effectiveness of C primary afferent fibers of different origins in evoking a prolonged facilitation of the flexor reflex in the rat, J. Neurosci., 6 (1986) 1433–1442.

[23] Young, M.R., Fleetwood-Walker, S.M., Mitchell, R. and Munro, F.E., Evidence for a role of metabotropic glutamate receptors in sustained nociceptive inputs to rat dorsal horn neurons, Neuropharmacology, 33 (1994) 141–145.

THE ROLE OF TACHYKININS AND SOMATOSTATIN IN SPINAL NOCICEPTIVE PROCESSING

S.M. Fleetwood-Walker, R.M.C. Parker, F. Munro, M. Young, P.J. Hope and R. Mitchell;*
Department of Preclinical Veterinary Sciences, University of Edinburgh, Edinburgh;
**M.R.C. Brain Metabolism Unit, Edinburgh*

SUMMARY

Since not only substance P (SP) but also neurokinin A (NKA) appears to be released from fine primary afferents upon noxious cutaneous stimulation we have addressed the role of their preferential targets (NK_1 and NK_2 receptors respectively) in mediating nociceptive responses of spinal dorsal horn neurons. By making extracellular recordings of neurons in anaesthetised rats (and cats) whilst applying drugs locally by ionophoresis it was shown that NK_2 agonists facilitate and NK_2 antagonists prevent responses to brief thermal noxious stimuli in a highly selective manner. NK_1 receptor drugs had no marked effect, although NK_1 agonists could readily excite laminae IV/V but not lamina I neurons. NK_2 agonists excited both deep and superficial cells, apparently producing a selective desensitising block of thermal nociception in lamina I.

NK_1 and NK_2 receptor drugs were also assessed in two models of sustained C-afferent input associated with peripheral inflammation, hyperalgesia and increased responsiveness of dorsal horn. Neuronal activity wind-up over tens of minutes by cutaneous application of mustard oil was inhibited by NK_2 but not NK_1 antagonists. Expression of mRNA for preprodynorphin elicited over 6 hours by intraplantar injection of λ-carrageenan was similarly affected.

Experiments with another peptide released from fine nociceptive afferents, somatostatin (SS) indicated that just like NK_2 receptors, the SS_2 subtype of SS receptor may well be an essential component in mediation of brief thermal nociceptive inputs.

The interrelations between the roles played by NK_2, SS_2 and NK_1 receptors (and indeed receptors for other factors co-released from nociceptive afferents) will be important to elucidate.

INTRODUCTION

For a number of years there has been strong interest in the possibility that substance P (SP) is a transmitter mediating the input from fine primary afferent C fibres to dorsal horn neurons. In support of this hypothesis there is a body of early work describing prominent excitatory effects of SP on dorsal horn neurons, especially those responding to nociceptive inputs (see [1] for review). More recent work with in vitro preparations describes facilitation by SP of responses to excitatory amino acids [2]. In behavioural experiments, intrathecally-applied SP and NK_1 receptor agonists elicit a localised biting/scratching response, perhaps consistent with irritation or pain [3] while some but not all NK_1 antagonists produce behavioural analgesia [4,5]. However, when the release of tachykinins into dorsal horn has been examined by antibody microprobe or push-pull cannula techniques, it is clear that SP is released by noxious levels of mechanical stimulation but surprisingly not by noxious thermal stimulation at temperatures which selectively activate polymodal C-afferents [6,7]. Higher skin temperatures however which are likely to cause inflammatory damage, do release SP [8]. In contrast, both noxious pinch and heat cause release of neurokinin A (NKA) which originates in dorsal horn very largely from primary afferents [9] where it is generated by alternative splicing of the mRNA for the SP precursor [10].

At the level of sensory responses of single neurons or nociceptive reflexes there has been relatively little decisive data implicating tachykinins as mediators of nociception; due in large part to the low specificity and potency of the early antagonists. Although SP was reported [11] to excite only those neurons that received nociceptive inputs, this has not been observed by other workers [12]. Only recently with the advent of non-peptide NK_1 antagonists has solid evidence been presented for an actual role of NK_1 receptors in mediating responses of single neurons to high intensity afferent stimulation or peripheral noxious stimuli [13,14]. More extensive studies are needed because these new agents have been found to exert prominent and very relevant effects on Ca^{2+} channels [15]. Facilitation of a flexor reflex elicited either by SP or by conditioning stimulation of sural nerve was prevented by an improved peptide antagonist, Spantide II (a more potent blocker of NK_1 than NK_2 or NK_3 receptors) [16].

Our earlier studies, carried out on single neurons of anaesthetised cats, pointed clearly to a role of NK_2 rather than NK_1 receptors in mediating brief responses to noxious thermal stimuli [17]. NKA and other NK_2 agonists specifically facilitated such responses, whereas the early NK_2 antagonists [D-Pro^4,Lys^6,D-Trp^{7,9,10},Phe^{11}]SP_{4-11} and [D-Tyr^4,D-Trp^{7,9},Nle^{11}]SP_{4-11} abrogated thermal nociceptive responses, reversed the effect of NKA on these responses and demonstrated behavioural analgesia in rat tail flick and hot plate

tests. Interestingly, selective NK_3 agonists had no effect on dorsal horn neurons in those studies whereas NK_1 agonists merely inhibited nonnociceptive responses, without modifying thermal or mechanical nociceptive responses; despite clear evidence [6] that noxious pinch releases SP into dorsal horn.

The experiments described here were carried out to further investigate the relative importance of NK_1 and NK_2 receptors in spinal nociceptive processing. There is evidence that ligand binding sites and mRNA corresponding to both NK_1 and to a lesser extent NK_2 receptors are present in superficial dorsal horn [18-20].

METHODS

Experiments were carried out in chloralose/urethane-anaesthetised rats examining the responses of single dorsal horn neurons during localised ionophoresis of NK receptor antagonists and employing a range of sensory stimuli. Some experiments, particularly comparing the effects of tachykinins and somatostatin (SS; shown to be released into dorsal horn by noxious, but not damaging levels of thermal stimuli [6,7]) were carried out in cats. The detailed methods, and sources and specificity of the relevant pharmacological agents are described in the publications indicated.

RESULTS

In the first series of experiments, novel and highly selective NK_1 and NK_2 antagonists were tested on the nociceptive/non-nociceptive responses of rat laminae IV/V neurons [21,22]. In 17 out of 19 neurons tested with the NK_2 antagonist L-659874 there was clear and selective inhibition of thermal (but not mechanical) nociceptive responses. In contrast, the NK_1 antagonists L-668169 and GR 82334 were without effect on nociception in 6 out of 8 and 4 out of 6 cases respectively, yet caused facilitation of non-nociceptive responses in over 75% of the cells. It seems clear that not only in cat but also in rat, NK_2 receptors play an important role in thermal (if not mechanical) nociception.

One possible reason for the lack of forthcoming evidence for a role of NK_1 receptors is that they are not present at a location accessible to the drugs as applied. To address this issue we carried out experiments with agonists. Drugs were applied ionophoretically close to laminae IV/V neurons and as a comparison, lamina I neurons [22,23]. The ascending cells of lamina I were of interest for two reasons: (i) they are reported to show a greater predominance of nociceptive cells and (ii) they are reported to receive direct synaptic contacts from fine primary afferents (including some SP reactive terminals). In laminae IV/V, selective NK_1 and NK_2 agonists produced clear excitatory effects of a similar order of magnitude which gradually declined despite continued ionophoresis. In lamina I, NK_2 agonists reliably produced a marked yet transient excitation and the declining phase of this (desensitisation?) coincided remarkably with a selective diminution of responses to noxious heat. NK_1 agonists were without detectable effect in lamina I cells (Table 1).

Agonist	Fraction of cells excited (mean fold increase in firing rate)	
	Lamina I	*Laminae IV/V*
[Ac-Arg^6,Sar^9,Met(O2)^{11}]SP$_{6-11}$ (NK_1)	-	6/6 (x5.4 fold)
[Met(OMe)^{11}]SP (NK_1)	2/11 (<0.6 fold)	4/4 (x3.9 fold)
NKA (NK_2)	9/11 (x11.2 fold)	6/8 (x3.5 fold)
GR 64349 (NK_2)	7/9 (x7.8 fold)	5/5 (x4.0 fold)

Experiments were carried out in anaesthetised rats and drugs were applied by microionophoresis (10-80 nA for 3-12 min) from side barrels of the multibarrelled extracellular recording electrode.

Table 1. Effects of NK receptor agonists on spontaneous electrical activity of multireceptive neurons in lamina I and laminae IV/V.

Since the sensory models investigated relied on very brief (10 sec) cutaneous stimuli, we carried out further experiments utilising models of sustained inflammatory nociceptive input to ascertain whether these in contrast would provide evidence for a role of NK_1 over NK_2 receptors. Two models were selected: (i) electrical recording of cells during topical application of mustard oil (a C-fibre selective chemical irritant) to the cutaneous receptive field, and (ii) measurement of the expression of preprodynorphin (PPD) mRNA in dorsal horn neurons (using in situ hybridisation histochemistry) following localised inflammation induced by intraplantar injection of λ-carrageenan. These models provide sustained C-afferent input for relatively prolonged periods and covered the experimental time domains of tens of minutes and six hours respectively. NK receptor antagonists were administered locally to the spinal dorsal horn by ionophoresis in each case (Table 2). In the mustard oil model, several NK_2 but not NK_1 antagonists again prevented mustard oil-induced excitation of spontaneous neuronal activity [24,25]. In the carrageenan model, the same profile was apparent [26,27] with no discernible evidence for a role of NK_1 receptors. It is possible that more profound or qualitatively different inflammatory stimuli would reveal a contribution from NK_1 receptors, but (at least in the present experimental conditions) it appears that NK_2 receptors have a more prominent role in mediating nociception. Thus, there is evidence mainly from release studies to suggest that NK_1 receptors may play a greater role in nociception elicited by prolonged mechanical stimuli, noxious cold or formalin [6,7,28,29].

Since release experiments [6,7] have shown that in contrast to SP, SS (as well as NKA [9]) is released into dorsal horn by noxious heat, we compared the effects of agonists and antagonists for neurokinin [17,21,22] and somatostatin [30] receptors on brief sensory responses of laminae IV/V neurons (Table 3). Whilst SS-14 had broad antinociceptive effects on responses

Antagonist	Proportion of neurons inhibited	Mean percentage inhibition in affected cells (± s.e. mean)
L 668169 (NK_1)	1/11	-
GR 82334 (NK_1)	3/11	(31 ± 14)
L 659874 (NK_2)	23/24	50 ± 7
MEN 10376 (NK_2)	4/4	38 ± 6
R 396 (NK_2)	3/3	44 ± 10

Electrical activity of multireceptive laminae IV/V neurons was recorded (as in Table 1) during topical application of mustard oil (5-20% in paraffin oil); a C-fibre selective chemical irritant [24,25]. One to five repeated applications over 10-15 min caused marked increases in firing rate (138 +/- 33 fold of background, mean +/- s.e. mean) which were sustained for more than 20 min and allowed testing of NK antagonists by ionophoresis (10-80 nA for 1-6 min).

a) Mustard oil-induced electrical activity.

Antagonist	Number of PPD mRNA expressing laminae I cells (% of contralateral controls; mean ± s.e.mean)	PPD mRNA silver grain density in expressing cells (% of contralateral controls; mean ± s.e.mean)
L 668169 (NK_1)	98 ± 7	94 ± 7
GR 82334 (NK_1)	103 ± 8	113 ± 8
L 659874 (NK_2)	68 ± 5	46 ± 6

The expression of preprodynorphin mRNA in lamina I neurons of anaesthetised rats subjected to bilateral intraplantar injection of the inflammatory agent λ-carrageenan (150µl of 1.4% in saline; n=12) was measured by in situ hybridisation histochemistry [26,27]. Neurokinin receptor antagonists were continuously ionophoresed unilaterally into the superficial dorsal horn of spinal segments shown to display corresponding receptive field areas. Drugs were ejected (50nA) for 1 hour prior to and then throughout the 6 hours of carrageenan stimulation. Drug effects were observed over about 700µm centred on the ejection site; the data shown are for the 200µm in closest proximity to the electrode.

b) Carrageenan-induced PPD mRNA expression

Table 2. Effects of NK receptor antagonists on the responses of dorsal horn neurons in models of sustained inflammatory nociception.

Drug (Predominant target)	Predominant effects on sensory responses (proportion of cells so influenced) Noxious heat	Noxious pinch	Innocuous brush
a) Agonists			
SS-14 (SS_1 > SS_2)	≥(9/11)	≥(5/5)	0(10/12)
SS-28 (SS_2 > SS_1)	"(5/6)	0(5/5)	0(6/6)
[D-Trp^8,D-Cys^{14}]SS-14 (SS_2 > SS_1?)	"(5/6)	0(5/6)	0(5/6)
[Met-Ome^{11}]SP (NK_1 > NK_2)	0(13/16)	0(6/9)	≥(12/16)
NKA (NK_2 > NK_1)	"(17/18)	0(11/14)	0(16/18)
b) Antagonists			
cyclo-[7-aminoheptanoyl, Phe,D-Trp,Lys,O-benzyl-Thr] (SS_1/SS_2?)	≥(7/10)	0(8/10)	0(10/11)
[D-Arg^1,D-Trp^{7,9},Leu^{11}]SP (NK_1 > NK_2)	0(10/12)	0(9/10)	0(10/12)
[D-Pro^4,Lys^6,D-Trp^{7,9,10}, Phe^{11}]SP_{4-11} (NK_2 > NK_1)	≥(11/13)	0(8/9)	0(11/13)

Experiments were carried out as described previously [17,30] on anaesthetised cats, applying drugs by microionophoresis from a multibarrelled electrode located in lamina II immediately dorsal to the recording electrode. Since the required diffusion distances in the cat are much greater than in the rat, ionophoresis was at higher currents (350–400 nA for 6-15 min), which in ejection of 1M NaCl were shown to have no discernible effect alone on neuronal activity.

Table 3. Effects of somatostatin and neurokinin receptor agonists and antagonists on sensory responses of multireceptive laminae IV/V neurons.

to both thermal and mechanical noxious stimuli (just as in rat [30]), SS-28 and [D-Trp^8, D-Cys^{14}]SS-14 selectively facilitated solely thermal nociceptive responses. This is analogous to the effect of the NK_2 agonist NKA and the parallel between effects of NKA and SS-28 is further indicated by the identical effects of their antagonists ([D-Pro^4,Lys^6,D-Trp^{7,9,10},Phe^{11}]SP_{4-11} and cyclo-[7-aminoheptanoyl,Phe,D-Trp,Lys-O-benzyl-Thr] respectively) in selective abrogation of thermal nociception. As in the other experimental models described above, there was little evidence for a significant role of NK_1 receptors.

DISCUSSION

The present results provide strong support for a functionally-important role of dorsal horn NK_2 receptors in thermal nociception. Although present at much lower levels than NK_1 receptors, both ligand binding sites and mRNA for NK_2 receptors are present in spinal cord [18,20]. In support of our contention, the flexor reflex facilitation elicited by NKA or by conditioning stimulation of the gastrocnemius nerve is prevented by selective NK_2 antagonists [31]. Furthermore, the excitation of dorsal horn neurons brought about by capsaicin applied to the dorsal roots of an in vitro spinal cord/dorsal root preparation is similarly prevented by NK_2 and not NK_1 antagonists [32].

NK_1 receptors may be more important in nociception which may rely more on different classes of fine afferent inputs, for example high threshold mechanoreceptive afferents in prolonged and severe pinch-mediated responses. Both noxious cold [7] and profoundly damaging inflammatory stimuli such as intraplantar formalin [29] or ultraviolet radiation-induced burning [33] may invoke a greater NK_1 contribution.

It is clear that the two main processing variants from preprosomatostatin (SS-14 and SS-28) exert quite different effects on somatosensory processing. Both are present in dorsal horn [34], show selectivity for the two subtypes of SS receptor (SS_1 and SS_2, respectively [35]) and have been shown in other CNS regions to exert quite distinct electrophysiological effects [36]. It appears that just like NKA, SS-28 may act as a transmitter of thermal nociceptive afferents. Whilst the antagonist experiments show each is necessary for maintained mediation of inputs, the full range of consequences of a thermal nociceptive input may not be felt by dorsal horn neurons unless they receive combined signals from NKA, SS-28 and possibly other co-released factors.

ACKNOWLEDGEMENTS

This work was supported primarily by the Wellcome Trust. We are grateful to Phil Birch, Les Iversen, Domenico Regoli and Carlo Maggi for gifts of compounds.

REFERENCES

1. Fleetwood-Walker, S.M. and Mitchell, R. Role of substance P in nociception. *Current Opinion in Anaesthesiol.* 1989; 2: 645-648.
2. Rusin, K.I., Ryu, P.D. and Randic, M. Modulation of excitatory amino acid responses in rat dorsal horn neurons by tachykinins. *J. Neurophyisol.* 1992, 68: 265-286.
3. Hylden, J.L.K. and Wilcox, G.L. Intrathecal substance P elicits a caudally-directed biting and scratching behaviour. *Brain Res.* 1981; 217: 212-215.
4. Post, C. and Folkers, K. Behavioural and anti-nociceptive effects of intrathecally-injected substance P analogues in mice. *Europ. J. Pharmacol.* 1985; 113: 335-342.
5. Lecci, A., Giuliani, S., Patacchini, R., Viti, G. and Maggi, C.A. Role of NK1 tachykinin receptors in thermonociception: effect of (+/-) CP96,345, anon-peptide substance P antagonist on the hot plate test in mice. *Neurosci. Lett.* 1991; 129: 299-302.
6. Kuraishi, Y., Hirota, N., Sato, Y., Hanashima, N., Tagaki, H. and Satoh, M. Stimulus specificity of peripherally-evoked substance P release from the rabbit dorsal horn in situ. *Neuroscience* 1989; 30: 241.
7. Tiseo, P.J., Adler, M.W. and Liu-Chen, L.-Y. Differential release of substance P and somatostatin in the rat spinal cord in response to noxious cold and heat: effect of dynorphin A(1-17). *J. Pharmacol. Exptl. Therap.* 1990; 252: 539-545.
8. Duggan, A.W., Hendry, I.A., Morton, C.R., Hutchison, W.D. and Zhao, Z.Q. Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. *Brain Res.* 1988; 451: 261.
9. Ogawa, T., Kanazawa, I. and Kimura, S. Regional distribution of substance P, neurokinin A and neurokinin B in rat spinal cord, nerve roots and dorsal root ganglia, and the effects of dorsal root or spinal transection. *Brian Res.* 1985; 359; 152-157.
10. Nawa, H., Kotani, H. and Nakanishi, S. Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing. *Nature* 1984; 312: 729-734.
11. Henry, J.L. Effects of substance P on functionally-identified units in cat spinal cord. *Brain Res.* 1976; 114: 439-451.
12. Hill, R.G., Salt, T.E. and Leighton, G.E. Actions of putative substance P antagonists on neurones in the CNS. In:Hakanson, R. and Sundler, F., eds. Tachykinin Antagonists. Amsterdam, Elsevier: 1985; 377-382.
13. De Koninck, Y. and Henry, J.L. Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons in vivo by noxious stimulation. *Proceedings of National Acad. Sciences* USA 1991; 88: 11344.
14. Radhakrishnan, V. and Henry, J.L. Novel substance P antagonist, CP 96345, blocks responses of cat spinal dorsal horn neurons to noxious cutaneous stimulation and to substance *P. Neurosci. Lett.* 1991; 132: 39-43.
15. Schmidt, A.W., McLean, S. and Heym, J. The substance P receptor antagonist CP 96345 interacts with Ca2+ channels. *Europ. J. Pharmacol.* 1992; 215: 351-355.
16. Wiesenfeld-Hallin, Z., Xu, X.-J., Hakanson, R., Feng, D.-M. and Folkers, K. The specific antagonistic effect of intrathecal Spantide II on substance P- and C-fibre conditioning stimuli-induced facilitation of the nociceptive flexor reflex in rat. *Brain Res.* 1990; 526: 284.
17. Fleetwood-Walker, S.M., Mitchell, R., Hope, P.J., El-Yassir, N., Molony V. and Bladon, C.M. The involvement of neurokinin receptor subtypes in somatosensory processing in the superficial dorsal horn of the cat. *Brain Res.* 1990: 519: 169-182. 1992; 579: 357
18. Yashpal, K., Dam, T.V. and Quirion, R. Quantitative autoradiographic distribution of multiple neurokinin binding sites in rat spinal cord. *Brain Res.* 1990; 506: 259.
19. Tsuchida,K., Shigemoto,R., Yokata,Y. and Nakanishi, S. Tissue distribution and quantitation of the mRNAs for three tachykinin receptors. *Europ. J. Biochem.* 1990; 193: 751-757.
20. Takeda, Y. and Krause, J.E. Pharmacological and molecular biological studies on the diversity of rat tachykinin NK-2 receptor subtypes in rat CNS, duodenum, vas deferens and urinary bladder. *Annals of the New York Academy of Sciences* 1991; 632: 479.
21. Fleetwood-Walker, S.M., Hope, P.J., Parker, R.M.C. and Mitchell, R. Effects of antagonists selective for neurokinin-1 and neurokinin-2 receptors on somatosensory responses of rat dorsal horn neurons. *British Journal of Pharmacology* 1991; 104: 472.
22. Fleetwood-Walker, S.M., Parker, R.M.C., Munro, F., Hope, P.J. and Mitchell, R. Evidence for a role of NK-2 receptors in mediating brief nociceptive inputs to rat dorsal horn (laminae III-V) neurons. *European Journal of Pharmacology* (submitted).
23. Fleetwood-Walker, S.M., Parker, R.M.C., Munro, F., Mitchell, R. and Birch P. Excitation of rat dorsl horn laminae I neurons by selective agonists at NK2 but not NK1 receptors. *Neuropeptides* 1992; 22: 23.
24. Munro, F.E., Fleetwood-Walker, S.M., Parker, R.M.C. and Mitchell, R. Mustard oil-evoked activation of rat dorsal horn neurons is inhibited by NK2 but not NK1 receptor antagonists. *Brit. J. Pharmacol.* 1993; 108: 243P.
25. Munro, F.E., Fleetwood-Walker, S.M., Parker, R.M.C. and Mitchell, R. The effects of neurokinin receptor antagonists on mustard oil-evoked activation of rat dorsal horn neurons. *Neuropeptides* 1993 (in press).
26. Parker, R.M.C., Fleetwood-Walker, S.M., Rosie, R., Munro, F.E. and Mitchell, R. Effects of neurokinin receptor antagonists on carrageenan-induced expression of preprodynorphin mRNA in rat dorsal horn lamina I neurons. *Brit. J. Pharmacol.* 1993; 108: 289P.

27. Parker, R.M.C., Fleetwood-Walker, S.M., Rosie, R., Munro, F.E. and Mitchell, R. Inhibition by NK2 but not NK_1 antagonists of carrageenan-induced preprodynorphin mRNA expression in rat dorsal horn lamina I neurons. *Neuropeptides* (submitted).
28. Ohkuba, R., Shibata, M., Takahashi, H. and Inoki, R. Roles of substance P and somatostatin in transmission of nociceptive information induced by formalin in spinal cord. *J. Pharmacol. Expt. Therap.* 1990; 252: 1261-1268.
29. Takagi, H and Kuraishi, Y. Substance P, somatostatin and pain transmission. *Taisha* 1986; 23: 33-37.
30. Fleetwood-Walker, S.M., Hope, P.J., Molony, V., Parker, R.M.C., Munro, F. E. and Mitchell, R. Distinct effects of somatostatin-14 and somatostatin-28 on nociceptive responses of spinal dorsal horn neurons. *Brit. J. Pharmacol.* 1992; 105: 132P.
31. Xu, H.-J., Maggi, C.A. and Wiesenfeld-Hallin, Z. On the role of NK2 tachykinin receptors in the mediation of spinal reflex excitability in the rat. *Neurosci.* 1991; 44: 483-489.
32. Urban, L., Maggi, C.A., Nagy, I. and Dray, A. The selective NK2 receptor antagonist MEN 10376 inhibits synaptic excitation of dorsal horn neurons evoked by C-fibre activation in the in vitro spinal cord. *Neuropeptides* 1992; 22: 68.
33. Thompson, S.W.N., Urban, L. and Dray, A. NMDA and tachykinin receptor-mediated contributions to the C-fibre-evoked response in the neonatal rat spinal cord in vitro are enhanced following peripheral inflammation. *Brit. J. Pharmacol.* 1993; 108: 22P.
34. Tessler, A., Himes, B.T., Gruber-Bollinger, J. and Reichlin, S. Characterisation of forms of immunoreactive somatosatin in sensory neurons and in normal and deafferented spinal cord. *Brain Res.* 1986; 370: 232-240.
35. Raynor, K. and Reisine, T. Subtypes of somatostatin receptors are expressed in the anterior pituitary cell line GH3. *J. Pharmacol. Exptl. Therap.* 1993; 264: 110-116.
36. Wang, H.-L., Bogen, C., Reisine, T. and Dichter, M. Somatostatin-14 and somatostatin-28 induce opposite effects on K+ currents in rat neocortical neurons. *Proc. Natl. Acad. Sci. (USA)* 1989; 86: 9616-9620.

Proceedings of the 7th World Congress on Pain, Progress in Pain Research and Management, Vol. 2, edited by G.F. Gebhart, D.L. Hammond, and T.S. Jensen, IASP Press, Seattle, © 1994.

23

Receptor and Cellular Mechanisms Involved in Mustard Oil–Induced Activation of Dorsal Horn Neurons

Fiona E. Munro,[a] Marie R. Young,[a]
Susan M. Fleetwood-Walker,[a]
Rachel M.C. Parker,[a] and Rory Mitchell[b]

[a]*Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh, and* [b]*MRC Brain Metabolism Unit, Edinburgh, UK*

INTRODUCTION

For many years substance P (SP) has been a prominent candidate for the transmitter of primary afferent nociceptive fibers (Henry 1976). Although considerable evidence suggests a role for SP (and NK_1 receptors) in nociceptive transmission (Coderre et al. 1993; Maggi et al. 1993), it is unlikely to be the only relevant transmitter. SP together with neurokinin A (NKA) and excitatory amino acids (EAAs) are released into the dorsal horn by noxious or high-intensity stimulation of afferents (Duggan et al. 1990; Skilling et al. 1988; Petermann et al. 1986). Evidence from both electrophysiological and reflex studies suggests that transmitters such as NKA and EAAs also may be crucial to nociception through their actions at NK_2, NMDA, and non-NMDA receptors (Coderre et al. 1993).

Several lines of evidence point to a role for NKA in nociceptive transmission. Ligand binding (Yashpal et al. 1990) and mRNA hybridization studies (Takeda and Krause 1991) have confirmed that there are NK_2 receptors in the spinal cord. NKA is present in primary sensory neurons (Ogawa et al. 1985) and is released by capsaicin from primary afferent terminals (Saria et al.

1986). In a model of acute nociception, we have previously shown that responses of dorsal horn neurons to brief thermal (but not mechanical) r ious cutaneous stimuli are inhibited by selective NK_2 but not NK_1 rece antagonists (Fleetwood-Walker et al. 1990, 1992a). Also in an in vitro sp cord/dorsal root preparation, neuronal activity evoked by brief applicatio capsaicin to the dorsal root ganglion is inhibited by a selective NK_2 antago (Urban et al. 1992). Following both noxious mechanical and noxious ther stimuli, release of NKA-immunoreactive material is detected throughout dorsal horn in a diffuse pattern (Duggan et al. 1990; Hope et al. 1990) intrathecal administration of NKA gives rise to behavioral responses indi tive of pain (Cridland and Henry 1986; Fleetwood-Walker et al. 1990).

The EAA glutamate is present at high levels in local interneurons of dorsal horn (Rizzoli 1968; Fagg and Foster 1983; Storm-Mathisen and Otter 1987) as well as in primary afferent terminals, as demonstrated immunolabeling of granular vesicles following rhizotomy (DeBiasi and Rusti 1988). Upon noxious cutaneous stimulation or low- or high-intensity elec cal stimulation to peripheral nerves, glutamate is released (Skilling et 1988), where it may act via several receptors (Schoepp et al. 1990a; Watk et al. 1990). The ionotropic group of receptors, including α-amino-3-hydro 5-isoxazole-4-propionate (AMPA) and N-methyl-D-aspartate (NMDA) typ have been extensively studied and it is now well established that the NMI receptor is involved in hypersensitized states such as long-term potentiat (LTP) in the hippocampus (Collingridge 1992) and wind-up in the spinal c (Davies and Lodge 1987; Dickenson and Sullivan 1987, 1990; Thompson al. 1990). A metabotropic group of glutamate receptors (mGlu), linked G-proteins, also may be involved in such transmission. Studies have dem strated a role for mGlu receptors, including a role in an NMDA recept independent model of LTP (Bortolotto and Collingridge 1993). Previous stud have demonstrated that 1-amino-cyclopentane-1,3-dicarboxylic acid (ACPI a metabotropic glutamate receptor agonist (Irving et al. 1990), can augm the actions of ionotropic agonist-induced activity in spinal cord preparatic (Cerne and Randic 1992; Bleakman et al. 1992), although the cellular mecl nism responsible has yet to be elucidated.

In our study, we used a prolonged noxious chemical stimulus to inve gate the involvement of NK_1, NK_2, mGlu receptors, and protein kinase (PKC) in the sustained activation of dorsal horn neurons. The model used topical cutaneous application of mustard oil (allyl isothiocyanate), a chemi irritant that selectively activates C (and occasionally some A-delta) affer fibers (Heapy et al. 1987).

METHODS

ELECTROPHYSIOLOGY

We conducted experiments on male Wistar rats (250–340 g) anaesthetized with intravenous α-chloralose (60 mg/kg) and urethane (1200 mg/kg) after induction with halothane. Supplementary doses of α-chloralose were given as required. Rectal temperature was continuously monitored and maintained between 37–38°C. The rats respired spontaneously, but oxygen was supplied to the area of the tracheal cannula to enrich the inspired air.

The thoraco-lumbar spinal column was supported by clamps under the lateral processes and a laminectomy (segments L1–L4) was performed. To increase the recording stability, a 2% agar solution was injected under the spinal bone at the rostral end of the laminectomy and then poured over the exposed cord. A core of agar was removed from above the recording region, the dura carefully cut, and a pool of liquid paraffin applied to the region.

Extracellular recordings were made via the central barrel (4 M NaCl pH 4.0–4.5) of a seven-barreled glass microelectrode. One side barrel contained 1 M NaCl (pH 4.0–4.5) for automatic current balancing and current controls. Another side barrel contained Pontamine Sky Blue (2% in 0.5 M sodium acetate) for determination of neuronal location by ejection at the recording site followed by histological examination. The other barrels of the electrode contained NK_1, NK_2 or mGlu receptor antagonists or PKC inhibitors, which were applied locally by ionophoresis over a range of currents (10–80nA) for several minutes. NK_1 (L668,169) and NK_2 (L659,874) antagonists were prepared as 0.15 mM solutions in distilled water with 0.3% dimethylformamide, and the NK_1 antagonist GR82334 was a 1 mM aqueous solution; all were pH 4.5–5.0. Metabotropic glutamate (mGlu) antagonists (L- and D-2-amino-3-phosphoproprionate, L- and D-AP3) and (RS)-4-carboxy-3-hydroxyphenylglycine (CHPG) were 10 mM aqueous solutions pH 8.0–8.5. PKC inhibitors chelerythrine (1 mM aqueous solution) and GF109203X (0.2 mM aqueous solution with 0.2% dimethylformamide) were pH 4.0.

All neurons were located in dorsal horn laminae IV/V, had excitatory receptive fields on the ipsilateral hindlimb (excluding glabrous skin), and were multireceptive (i.e., displayed responses to both noxious and innocuous stimuli). Neurons were initially found by their responsiveness to widespread manual brushing of the ipsilateral hindlimb. Cutaneous receptive fields of the

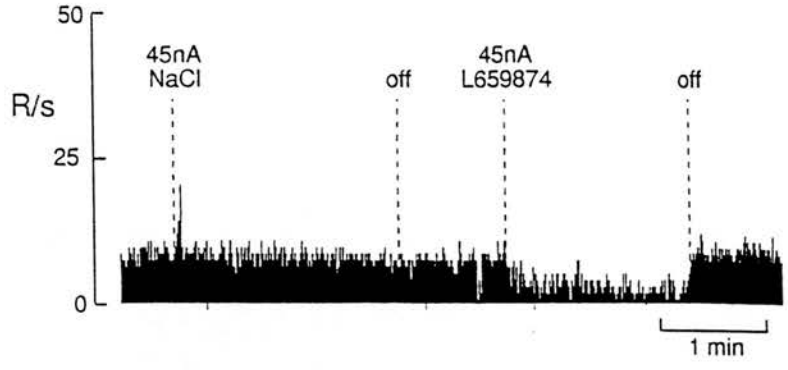


Fig. 1. Typical inhibitory effects of the NK_2 antagonist L659,874 on mustard oil–evoked activity of laminae IV/V dorsal horn neurons. Ionophoretic application of NaCl had no effect, whereas L659,874 consistently caused greater than 50% inhibition of evoked activity.

neurons were first localized by responses to manual brush, then the more restricted pinch and noxious heat fields were identified. The mustard oil (5–20% in paraffin oil) was applied topically with a paintbrush to the receptive fields.

BIOCHEMISTRY

To examine the subcellular translocation of protein kinase C (PKC, a facet of its activation in response to external stimuli), we performed the following [^{3}H]PDBu binding assay in spinal cord tissue ipsilateral/contralateral to mustard oil stimulation.

Male Wistar rats (273–330 g) were briefly anaesthetized with halothane, and mustard oil (7–20%) was applied three times to the left hindfoot and leg over a 15-minute period. This treated leg was bandaged to avoid cross-contamination and after one hour the rats were killed and the spinal cords removed and hemisected.

Each side of the spinal cord was separately homogenized in 0.4 ml of ice-cold buffer containing 20 mM Tris-HCl at pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA and 1 mM phenylmethylsulphonylfluoride with 0.01% leupeptin and 20 µM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64). The samples were then centrifuged at 25,000 × g for 20 minutes at 4°C. The supernatant and pellet were carefully separated and the pellet was rehomogenized in 0.3 ml Tris-BSA (50 mM Tris HCl, pH 7.4), with 4 mg/ml essential fatty acid–free bovine serum albumin. While membrane binding was carried out in Tris-BSA, cytosolic binding assays additionally contained 1 mM $CaCl_2$ and 75 mM magnesium acetate and 1.35 mg/ml sonicated

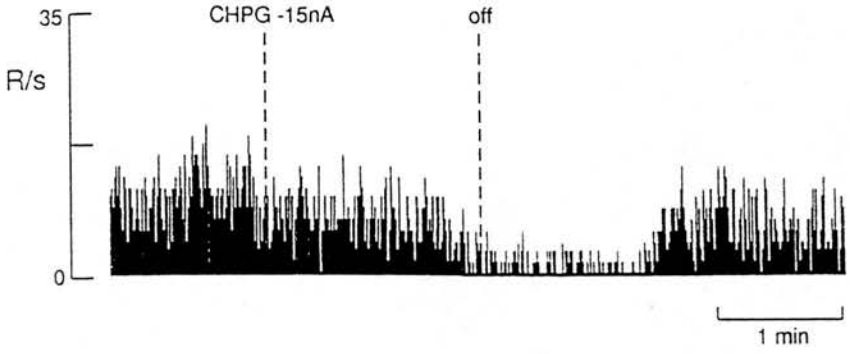


Fig. 2. An example of the inhibition of mustard oil–induced firing of a dorsal horn neuron by the metabotropic glutamate receptor antagonist CHPG. In this case recovery was observed within two minutes of termination of ionophoresis of the antagonist.

phosphatidylserine, sodium salt. Assays were conducted in a total volume of 275 µl (30 min, 37°C), with 5 nM [^{3}H]PDBu (approximately 0.03 µCi per tube) and dimethylformamide (0.5% final) or 10 µM PDBu in dimethylformamide for total and nonspecific binding measurements, respectively. Tissue samples gave a total binding of less than 10% of total radioactivity present. Protein was precipitated at 4°C by the addition of 100 µl of 12 mg/ml bovine gamma globulin and 300 µl of 24% polyethyleneglycol 8000 in 50 mM Tris-HCl (pH 7.4). After 20 minutes on ice, assay tubes were centrifuged (12,000 × g, 5 min, 4°C), the supernatant aspirated, and the [^{3}H] radioactivity in each pellet determined after solubilization.

All laboratory chemicals were from Sigma Chemical Co., Poole, Dorset, UK, except phosphatidyl serine from Lipid Products Ltd., Nutfield, Sussex, UK. Radioligand was from Du Pont NEN, Stevenage, Herts., UK. Drugs were from Cambridge Research Biochemicals Ltd., Northwich, Cheshire, UK; Calbiochem-Novabiochem, Nottingham, Notts., UK; Tocris Neuramin, Churchill Building, Langford House, Langford, Bristol, Avon, UK; or were gifts from Merck, Sharp and Dohme and Glaxo Group Research.

RESULTS

After application of the C-nociceptor-selective chemical algogen mustard oil, virtually all the multireceptive neurons showed a large and prolonged increase in activity (149 ± 36-fold of background, mean ± S.E.M.). In most cases, the steady elevated firing rate was achieved by repeated application of mustard oil 3–5 times to the same site over a period of 10–15 minutes.

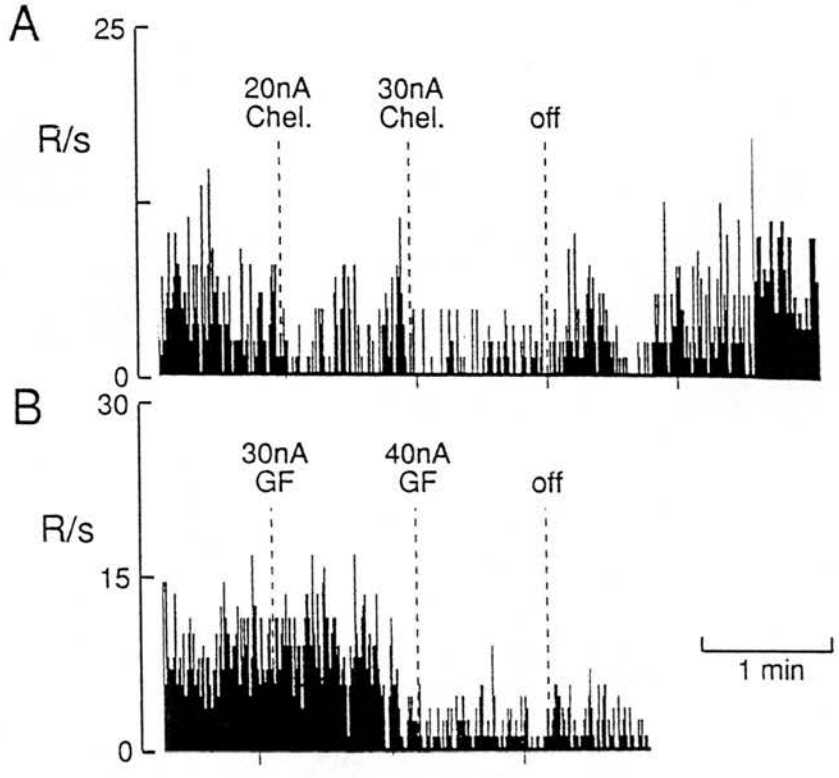


Fig. 3. The selective PKC inhibitors (A) chelerythrine and (B) GF109203X caused marked, yet recoverable inhibition of mustard oil–evoked activity.

The selective NK_2 antagonist L-659,874 (10–80 nA) caused a clear and maintained inhibition of the elevated activity, to 50 ± 7% mean ± S.E.M., of prior control level in 23 of 24 cells (Fig. 1). Recovery was observed in 13 of 17 cases examined after terminating ionophoresis of L659,874. In contrast, the two selective NK_1 antagonists L668,169 and GR82334 (20–80 nA) rarely inhibited mustard oil–evoked activity (in 1 of 11 and 3 of 11 cases, respectively). The mustard oil–evoked activity in many cells (n = 15) was unaffected by NK_1 antagonists but was inhibited by the NK_2 antagonist L659,874 (Munro et al. 1993).

Selective antagonists for mGlu (L-AP3 and CHPG) and NK_2 (L659,874) receptors also reliably inhibited the mustard oil–induced wind-up of neuronal activity. L-AP3 (–15 to –50 nA) and CHPG (–15 to –55 nA) inhibited the

Table I
Mustard oil–induced translocation of specific [^{3}H] PDBu binding sites
(% of total specific binding on control contralateral side)

	Cystolic Fraction	Membrane Fraction
Control (contralateral to mustard oil)	73.7 ± 2.5	26.2 ± 2.4
Test (ipsilateral to mustard oil)	37.6 ± 5.0	40.5 ± 4.8

Note: The proportion of [^{3}H] PDBu binding sites in the membrane fraction was significantly greater on the test side (mean 52%) than on the control side (mean 26%) $P < 0.05$, Wilcoxon test). Values are the means ± S.E.M. from nine separate experiments.

mustard oil–evoked activity (by 68 ± 7% and 56 ± 9% mean ± S.E.M.) in 11 of 16 and 7 of 8 cells, respectively (Fig. 2). Recovery was sometimes but not always observed. The stereoisomer of L-AP3, D-AP3 (which has a much lower affinity for mGlu receptors; Schoepp et al. 1990b) (–15 to –55 nA) had no effect in 5 of 5 cells.

The selective PKC inhibitors chelerythrine and GF109203X caused marked inhibition of mustard oil–induced activity in 8 of 9 and 8 of 8 cases respectively (Fig. 3). Rapid recovery was seen in the majority of cases after removal of the PKC inhibitor. In contrast, activity evoked by continuous innocuous brushing was completely unaffected by the PKC inhibitors (data not shown, $n = 5$).

In rats treated with mustard oil to one distal hindlimb, a significant translocation of specific [^{3}H]PDBu binding sites from the cytosolic to membrane fraction was observed in ipsilateral spinal segments L1–L5 in comparison with the contralateral (control) side (Table I).

DISCUSSION

These results indicate that both NK_2 and mGlu receptors play a crucial role in mediating the sustained excitation of lamina IV/V dorsal horn neurons elicited by cutaneous application of mustard oil. As mustard oil excites largely C-fibers (Heapy et al. 1987), this implies a role for NK_2 and mGlu receptors in the transmission of nociceptive information. For tachykinins, this finding agrees with our previous studies showing that ionophoretic administration of NK_2 but not NK_1 receptor antagonists inhibited the responses of both rat and cat dorsal horn neurons to brief noxious thermal stimuli applied to their cutaneous receptive fields (Fleetwood-Walker et al. 1990, 1991, 1992a,b).

This evidence for a role of mGlu receptors in sustained cutaneous nociceptive inputs that lead to hyperalgesia is novel and contrasts with data from Thompson et al. (1992) who found no evidence for inhibition by L-AP3 of

ventral root potentials evoked by high-intensity stimulation of dorsal roots in an in vitro spinal cord preparation. Despite several reports of the attenuation of wind-up by NMDA receptor antagonists (Davies and Lodge 1987; Dickenson and Sullivan 1987, 1990; Thompson et al. 1990), we found no evidence for attenuation of mustard oil–evoked responses by the D-isomer of AP3 (which is a weak antagonist at the NMDA receptor [Schoepp et al. 1990b]). This may simply be a consequence of low potency of the antagonist or it may indicate a genuine mechanistic difference in the present case.

We also carried out a preliminary investigation of the intracellular mechanisms underlying the sensitization of spinal dorsal horn neurons brought about by the repeated application of mustard oil. The role of PKC in this model was clearly indicated by two separate approaches: (1) the inhibition of electrophysiological responses by ionophoretic administration of selective PKC inhibitors, and (2) translocation/activation of PKC in response to mustard oil. Although many other mechanisms may contribute to the processing of sustained nociceptive inputs and the integral sensitization of dorsal horn neurons which they bring about, this preliminary evidence clearly identifies a crucial role of PKC. Since both NK_2 and some mGlu receptors bring about hydrolysis of phosphoinositides, it seems likely that either or both of these receptors could well contribute to PKC activation by means of elevation of cellular diglyceride contact.

REFERENCES

Bleakman, D., Rusin K.I., Chard P.S., Glaum S.R. and Miller R.J., Metabotropic glutamate receptors potentiate ionotropic glutamate responses in rat dorsal horn, Molec. Pharmacol., 42 (1992) 192–196.

Bortollotto, Z.A. and Collingridge, G.L., Characterisation of long term potentiation induced by the activation of glutamate metabotropic receptors in area CA1 of the hippocampus, Neuropharmacology, 32 (1993) 1–9.

Cerne, R. and Randic, M., Modulation of AMPA and NMDA responses in rat spinal dorsal horn neurons by trans-ACPD, Neurosci. Letts., 144 (1992) 180–184.

Coderre, T.J., Katz, J., Vaccarino, A.L. and Melzack, R., Contribution of central neuroplasticity to pathological pain: review of clinical and experimental evidence, Pain, 52 (1993) 259–285.

Collingridge, G.L., The mechanism of induction of NMDA receptor-dependant long-term potentiation in the hippocampus, Exp. Physiol., 77 (1992) 771–797.

Cridland, R.A. and Henry, J.L., Comparison of the effects of substance P, neurokinin A, physalaemin and eledoisin in facilitating a nociceptive reflex in the rat, Brain Res., 381 (1986) 93–99.

Davies, S.N. and Lodge D., Evidence for involvement of NMDA receptors in "wind-up" of class 2 neurons in the dorsal horn of the rat, Brain Res., 424 (1987) 402–406.

DeBiasi, S. and Rustioni A., Glutamate and substance P coexist in primary afferent terminals in superficial laminae of the spinal cord, Proc Nat Acad Sci.USA, 85 (1988) 7820–7824.

Dickenson, A.H. and Sullivan A.F., Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep dorsal horn nociceptive neurons following C fibre stimulation, Neuropharmacology, 26 (1987) 1235–1238

Dickenson, A.H. and Sullivan A.F., Differential effects of excitatory amino acid antagonists on dorsal horn nococeptive neurons in the rat, Brain Res., 506 (1990) 331–39.

Duggan, A.W., Hope, P.J., Jarrott, B., Schaible, H.G. and Fleetwood-Walker, S.M., Release, spread and persistence of immunoreactive NKA in the dorsal horn of the cat following noxious cutaneous stimulation, Studies with antibody microprobes, Neuroscience, 35(1) (1990) 195–202.

Fagg G.E. and Foster A.C., Amino acid neurotransmitters and their pathways in mammalian CNS, Neuroscience, 9 (1983) 701–719.

Fleetwood-Walker, S.M., Mitchell, R., Hope, P.J., El-Yassir, N., Molony, V. and Bladon, C.M., The involvement of neurokinin receptor subtypes in somatosensory processing in the superficial dorsal horn of the cat, Brain Res., 519 (1990) 169–182.

Fleetwood-Walker, S.M., Hope, P.J., Parker, R.M.C. and Mitchell, R., Effects of antagonists selective for neurokinin$_1$ and neurokinin$_2$ receptors on somatosensory responses of rat dorsal horn neurons, Br. J. Pharmacol., 104 (1991) 472.

Fleetwood-Walker, S.M., Mitchell, R., Hope, P.J., El-Yassir, N., Molony, V. and Bladon, C.M., The involvement of neurokinin receptor subtypes in somatosensory processing in the superficial dorsal horn of the cat: corrigendum, Brain Res., 579 (1992a) 357.

Fleetwood-Walker, S.M., Parker, R.M.C., Munro, F.E., Hope, P.J. and Mitchell, R., Evidence for a role of NK_2 receptors in mediating brief nociceptive inputs to rat dorsal horn (laminae III–V) neurons, Eur. J. Pharmacol., 242 (1992b) 173–181.

Heapy, C.G., Jamieson, A. and Russell, N.J.W., Afferent C fibre and A-delta activity in models of inflammation, Br. J. Pharmacol., 90 (1987) 164P.

Henry, J.L., Effects of substance P on functionally-identified units in cat spinal cord, Brain Res., 114 (1976) 439–451.

Hope, P.J., Lang, C.W. and Duggan, A.W., Persistence of immunoreactive neurokinins in the dorsal horn of barbiturate anaesthetised and spinal cats, following release by tibial nerve stimulation, Neurosci. Letts., 118 (1990) 25–28.

Irving A.J., Schofield J.G., Watkins J.C., Sunter D.C. and Collingridge G.L., 1S,3R ACPD stimulates and L-AP3 blocks calcium mobilization in rat cerebellar neurons, Eur. J. Pharmacol., 186 (1990) 363–365.

Maggi, C.A., Patacchini, R., Rovero, P. and Giachetti, A., Tachykinin receptors and tachykinin receptor antagonists, J. Auton. Pharmacol., 13 (1993) 23–93.

Munro, F.E., Fleetwood-Walker, S.M., Parker, R.M.C. and Mitchell, R., The effects of neurokinin receptor antagonists on mustard oil-evoked activation of rat dorsal horn neurons, Neuropeptides, 25 (1993) 299–305.

Ogawa, T., Kanazawa, I. and Kimura, S., Regional distribution of substance P, neurokinin a and neurokinin b in rat spinal cord, nerve roots and dorsal root ganglia, and the effects of dorsal root section or spinal transection, Brain Res., 359 (1985) 152–157.

Petermann, J., Fischer, J.A., Theodorsson-Norheim, E. and Lundberg, J.M. Simultaneous release of several tachykinins and calcitonin gene-related peptide from rat-spinal cord slices, Neurosci. Letts., 63 (1986) 310–312.

Rizzoli, A.A., Distribution of glutamic acid, aspartic acid, GABA and glycine in six areas of cat spinal cord before and after transection, Brain Res., 11 (1968) 11–18.

Saria, A., Gamse, R., Petermann, J., Fischer, J.A., Theodorsson-Norheim, E. and Lundberg, J.M., Simultaneous release of several tachykinins and calcitonin gene-related peptide from rat-spinal cord slices. Neurosci. Letts., 63 (1986) 310–312.

Schoepp, D.D., Bockaert, J. and Sladeczek, F., Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors, Trends in Pharmacological Sciences, 11 (1990a) 508–515.

Schoepp, D.D., Johnson, B.G., Smith E.C.R. and McQuaid, L.A., Stereoselectivity and mode of inhibition of phosphoinositide-coupled excitatory amino acid receptors by 2-amino-3-phosphopropionic acid, Molec. Pharmacol., 38 (1990b) 222–228.

Skilling, S.R., Smullin, D.H., Beitz, A.J. and Larson, A.A., Extracellular amino acid concentrations in the dorsal spinal cord of freely moving rats following veratridine and nociceptive stimula-

tion, J. Neurochem., 51 (1988) 127–132.
Storm-Mathisen, J. and Ottersen, O.P., Tracing of neurons with glutamate or GABA as putative transmitters, Biochem. Soc., 15 (1987) 210–213.
Takeda,Z. and Krause, J.E., Pharmacological and molecular biological studies on the diversity of rat tachykinin NK_2 receptor subtypes in rat CNS, duodenum, vas deferens and urinary bladder, Annals of the New York Academy of Sciences, 632 (1991) 479–482.
Thompson S.W.N., Gerber G., Sivilotti L.G. and Woolf C.J., Activity-dependent changes in rat ventral horn neurons in vitro: summation of prolonged evoked postsynaptic depolarizations produce D-APV sensitive wind-up, Eur. J. Neuroscience, 2 (1990) 638–649.
Thompson, S.W.N., Gerber, G., Sivilotti, G. and Woolf, C.J., Long duration ventral root potentials in the neonatal rat spinal cord in vitro: the effects of ionotropic and metabotropic excitatory amino acid receptor antagonists, Brain Res., 595 (1992) 87–97.
Urban, L. and Dray, A., Synaptic activation of dorsal horn neurons by selective C-fibre excitation with capsaicin in the mouse spinal cord *in vitro*, Neuroscience, 47 (1992) 693–702.
Watkins, J.C., Krogsgaard-Larsen, P. and Honore, T., Structure-activity relationships in the development of excitatory amino acid receptor agonists and antagonists, Trends in Pharmacological Sciences, 11 (1990) 25–27.
Yashpal, K., Dam, T.-V. and Quirion, R., Quantative autoradiographic distribution of multiple neurokinin binding sites in rat spinal cord, Brain Res., 506 (1990) 259–266.

Correspondence to: S.M. Fleetwood-Walker, PhD, Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK. Tel: 44-31-650-6091; Fax: 44-31-650-6576.